The Intradermal Skin Test in the Horse:
Value as a Diagnostic Modality in Equine Medicine

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(Abstract)

Recent studies have provided conflicting results in regards to equine intradermal skin testing and its use in defining causative antigens in IgE mediated diseases such as equine recurrent airway obstruction (RAO). This study was divided into two experiments. In the first experiment of this study, the hypothesis tested was normal horses would have minimal variability in the wheals formed by intradermal injection of positive control stimulants. This was evaluated by examining the repeatability of skin test wheals created by 5 concentrations of histamine, compound 48/80, and phaseolus vulgaris (PHA) within a normal horse and between 12 normal horses at 0.5 hours, 4 hours, and 24 hours post injection. Minimal variability was detected within individual horses and between 12 horses for histamine and compound 48/80 at 0.5 hours and for PHA at 4 hours. This information suggests that the intradermal injection of positive control substances is a repeatable test in normal horses.

In the second experiment of this study, the hypothesis tested was normal horses react differently to intradermal injection of positive control stimulants (histamine, compound 48/80, PHA) and/or an environmental antigen (*Aspergillus*) in comparison to horses affected with RAO. This was evaluated by identifying differences in wheal responses between normal horses and RAO affected horses. Concentration response curves were created in normal and RAO affected horses to the aforementioned stimulants at 0.5 hours, 4 hours, and 24 hours post injection. No statistically significant differences were noted in concentration response between normal and ROA affected horses when compound 48/80 and PHA were evaluated. RAO affected horses demonstrated a greater slope at the 0.5 hour time when compared to normal horses suggesting that RAO affected horses are

hypersensitive to intradermal injection of histamine. Injection of *Aspergillus* mix at 4000 protein nitrogen units/ml caused an intradermal wheal reaction at the 24-hour time in 4/5 RAO horses. This reaction was not noted in normal horses. This information suggests that there may be a positive relationship between causative antigens (i.e. *Aspergillus*) that may induce clinical RAO and positive intradermal skin test results.

An additional aspect that was evaluated in both experiments involved histologic examination of skin biopsies taken from wheals created by intadermal injection of histamine, compound 48/80, PHA, and *Aspergillus* at various times post injection. In the first experiment, intradermal injection of histamine caused severe dermal edema and margination of neutrophils and eosinophils at 0.5 hours. Compound 48/80 demonstrated mild to modest dermal edema at 0.5 hours while PHA demonstrated severe dermal edema, hemorrhage, and lymphactic ectasia at 4 and 24 hours. PHA also demonstrated a neutophilic inflammation at 4 hours that progressed to a mixed lymphohistiocytic and neutrophilic inflammation at 24 hours. In the second experiment, no edema and modest to moderate neutrophilic inflammation was noted in normal horses after intradermal injection of *Aspergillus* at 24 hours. In contrast, RAO affected horses demonstrated mild to modest edema and a mild to moderate mixed inflammatory response (lympho-histocytic, neutrophilic, eosinophilic) after intradermal injection of *Aspergillus* at 24 hours suggesting a delayed type response.

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Dedication

Dedicated with love to my faithful wife, Kristine, for her constant encouragement and understanding throughout this trying stage of our life and to my children, Olivia and Isabelle Wong, for their unconditional love and playfulness in times of stress.

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Chapter I: Introduction

Atopy and the Role of the Immune System: Overview

"Atopy", originating from the Greek atopos, meaning out of place, is often used to describe IgE-mediated allergic diseases. It has been defined as a genetically determined predisposition to develop sensitizing IgE antibodies to environmental antigens such weeds, grasses, trees, molds, and dust [1, 2]. Sensitizing IgE antibodies subsequently bind to mast cells in the skin or respiratory tract; secondary exposure to specific antigen(s) leads to cross-linking of mast cell bound IgE, mast cell degranulation, release of preformed and synthesized mast cell mediators, and the development of clinical allergy.

A number of inflammatory cells interact with one another and play collective and individual roles in the development of atopy. Both B and T lymphocytes are actively involved in the development of IgE mediated diseases. Through their interactions with antigen presenting cells (i.e. macrophages), T cells release specific cytokines that support B cell activation and the subsequent production of antibodies. In type I hypersensitivity disorders, B cells are induced to produce IgE antibodies; these IgE antibodies are intimately involved with mast cells and serve as an antigen receptor. Mast cells, when activated, release various mediators that cause smooth muscle contraction and chemotaxis of additional inflammatory cells such as neutrophils and eosinophils. Mast cell degranulation in the respiratory tract may cause clinical signs such as bronchoconstriction and hypersecretion of mucus and is commonly noted in conditions such as recurrent airway obstruction in horses [3]. Activated dermal mast cells may cause clinical signs of pruritis and uritcaria and are commonly noted in conditions such as

Culicoides hypersensitivity in horses [4, 5]. Clinical signs of atopy are therefore a culmination of complex inflammatory cell interactions and the various mediators that they release.

In recent literature, researchers have explored different theories that contribute to or cause atopic conditions in humans [6]. It has been demonstrated that there is an imbalance between specific T cell types in atopic individuals. T cells can be divided into $CD4^+$ or $CD8^+$ T cells based on cell surface markers and activity. All mature T cells express the CD3 receptor; in addition helper T cells express CD4 while cytotoxic and suppressor T cells express CD8. Stimulated naive $CD4^+$ T helper (T_h0) cells develop further into type 1 (T_h1) cells that secrete IFN-γ, IL-2, and TNF-β or type 2 (T_h2) cells that secrete IL-4, IL-5, IL-6, IL-10, and IL-13 [2, 6, 7]. T_h1 cells preferentially develop during bacterial infections and trigger phagocyte-mediated host defenses. Conversely, T_h2 cells predominate during immune reactions to environmental antigens and helminthic parasites and are responsible for phagocytic-independent host responses. Attention has been directed at the T cell subsets, T_h1 and T_h2 type cells, and the role they play in the development of allergic diseases.

The newborn immune response is dominated by T_h2 cells [6, 8]. Researchers have hypothesized that the T_h2 cell dominated environment in the normal newborn shifts to a T_h1 mediated environment in response to pathogen exposure as the individual ages. In the normal, non-atopic individual, the T_h1 dominated immunity produces minimal allergic reactions to antigens [8]. When a normal individual is exposed to common antigen they mount a low-grade immunologic response with the production of antigen-specific IgG antibodies under the influence of the T_h1 cell response. The T_h1 cell is

characterized by the production of interferon-γ, tumor necrosis factor-ß and IL-2 [6, 9-11]. In contrast, the atopic individual's immune system does not shift to the T_h1 mediated response but rather increases in the T_h2 mediated response. If the T_h2 cells predominate during immune responses to antigen, an exaggerated T_h2 cell response occurs with the release of various cytokines such as IL-4, IL-5, IL-9 and IL-13 by T_h2 cells. These cytokines, particularly IL-4 and IL-13, promote isotype switching of B cells with the subsequently production of allergen-specific IgE antibodies [6, 7, 10-15].

Under experimental conditions, T_h2 clones induced IgM, IgG, IgA, and IgE synthesis by B cells in the presence of specific antigen with the degree of response proportional to the number of T_h2 cells added to B cells [7]. Conversely, under the same conditions, T_h1 clones induced only IgM, IgG, and IgA synthesis [7]. The development of atopic conditions appears to involve the T_h2 cell response in which IgE plays a major role in the development of immediate Type I hypersensitivity responses by binding with receptors on mast cells and basophils.

The cytokines produced by the T_h2 cells, namely IL-4, IL-5, IL-9, and IL-13, appear to be intimately involved in the development of IgE mediated allergic inflammation. IL-4 regulates allergic inflammation by promoting T_h2 cell differentiation and IgE synthesis from B cells [16]. IL-4 also activates mast cells, induces hypersecretion of airway mucus, promotes cellular inflammation by induction of vascular cell adhesion molecule (VCAM-1) in asthmatic patients, and up regulates IgE receptors on mast cells, basophils, and B cells [16].

IL-5 is involved in eosinophilic inflammation and leads to increased eosinophil formation [17]. IL-5 is essential for maturation, growth and activation of eosinophils and

basophils within the bone marrow and subsequent release into the circulation. IL-5 also promotes eosinophil and basophil survival [18]. Several allergic diseases in humans such as nasal rhinitis, asthma, and atopic dermatitis have inflammatory responses characterized by eosinophilic infiltration [19]. Although there is not a strong relationship between eosinophilic inflammation and RAO in horses [20], eosinophils have been identified in biopsy samples of horses affected by *Culicoides* hypersensitivity [4, 21].

IL-13 has structural similarities to IL-4 and therefore expresses many of similar functions as IL-4. IL-13 promotes isotype switching by B cells to IgE synthesis, induces VCAM-1, and activates mast cells and eosinophils [22, 23]. However, IL-13 is not involved in T cell differentiation.

IL-9 has many functions that support the T_h2 type response by enhancing the activities of other cytokines. IL-9 enhances the IL-4 mediated production of IgE from B cells and acts synergistically with IL-5 to promote eosinophil maturation [24]. IL-9 also serves as a mast cell growth factor and promotes mast cells to express the high affinity IgE receptor FceRI. This later function may prime mast cells to respond to antigens via increased expression of IgE receptors [10, 24].

Atopy has been classified as a type I hypersensitivity reaction that requires at least three components: a disease-eliciting antigen (allergen); a transferable serum factor that discriminates an allergic patient from a normal patient (IgE); and a tissue component that is present in all individuals (mast cells) [25]. In type I hypersensitivity reactions, various antigens are absorbed percutaneously (via Langerhans' cells), inhaled (via dendritic cells), and/or ingested and provoke allergen-specific IgE or IgG production [26]. This occurs by translocation of the antigen to a regional lymph node by dendritic

cells/Langerhans' cells that subsequently act as antigen-presenting cells in the lymph node. Under the influence of two specific signals, isotype switching occurs and IgD/IgM producing B cells begin to produce antigen-specific IgE [1, 26]. The first signal is provided by the cytokines released from T_h2 cells, IL-4 and IL-13, while the second signal involves the interaction of CD40 ligand on the surface of T cells with CD40 receptor found on B cells [17]. These IgE antibodies circulate throughout the blood briefly before binding to high-affinity IgE receptors (FceRI) on the surface of mast cells in tissue or peripheral blood basophils. IgE also binds to low-affinity IgE receptors (FceRII or CD23) on the surface of lymphocytes, eosinophils, platelets, and macrophages [1, 27]. This primary/initial response to antigen is known as sensitization (Figure 4a). When an individual is later exposed to the same antigen, the antigen interacts with receptor bound IgE molecules and molecular bridging occurs with the FceRI receptors of sensitized mast cells and basophils. Activation and degranulation of these cells causes the immediate phase reaction characterized by the release of pharmacologically active compounds, primarily histamine and serotonin in the horse, causing vasodilation and smooth muscle contraction (Figure 4b).

In allergic patients with chronic atopy (i.e. chronic asthma, chronic atopic dermatitis), a late-phase reaction (>24 hours) may be observed [28-30]. This late-phase reaction is caused by the activation of antigen-specific T cells after hours to days of the immediate hypersensitivity reaction. The late-phase reaction is characterized by a strong T-cell infiltration and eosinophil activation in humans. It is speculated that antigen-specific IgE may have a role in activating antigen-specific T cells by facilitating antigen presentation (Figure 4c) [25].

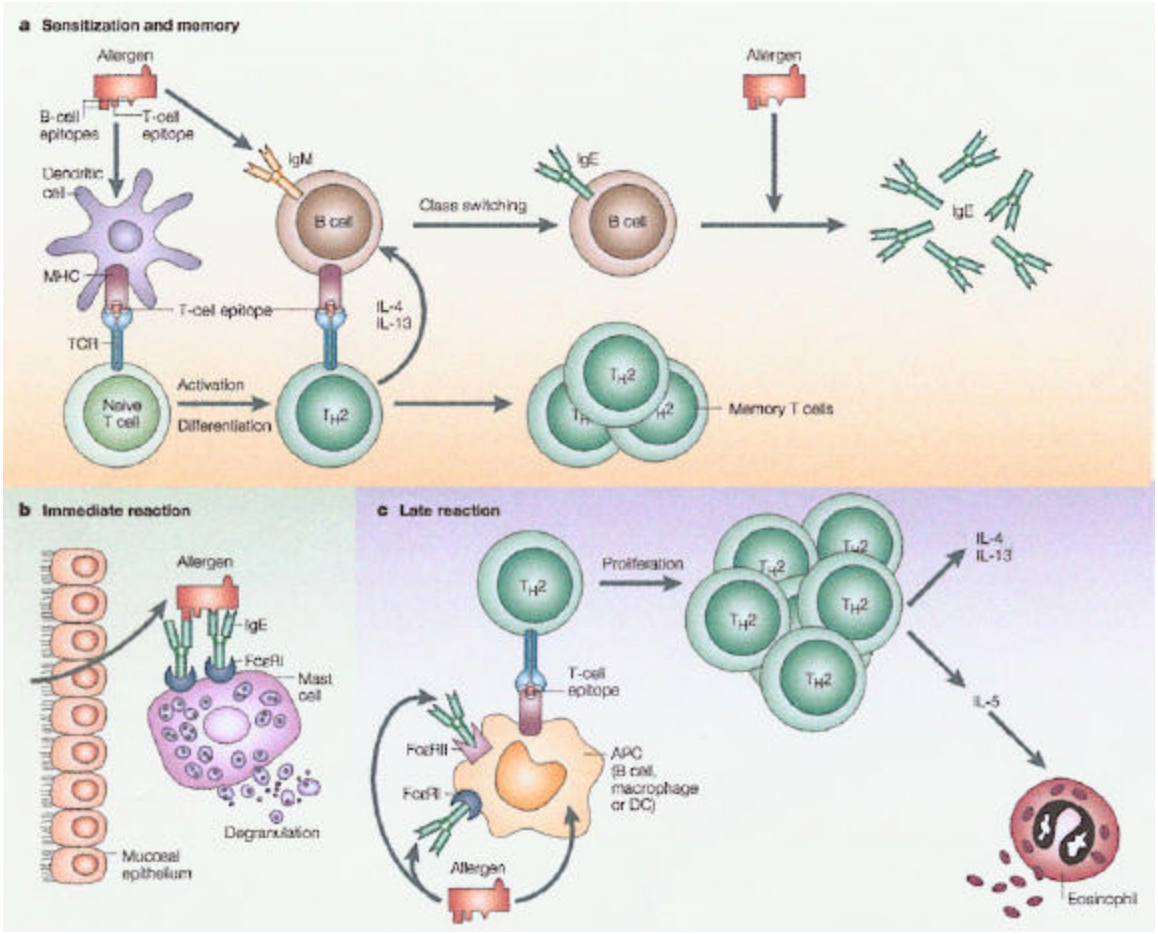

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Figure 1: Induction and maintenance of allergy.
a. Sensitization and memory. Initial contact with minute amounts of intact, soluble allergen at mucosal surfaces favors allergen uptake by potent antigen-presenting cells (i.e. dendritic cells) and/or immunoglobulin mediated capture by specific B cells. If T helper (T_h2) cell help is acquired, cytokines such as interleukin-4 (IL-4) and IL-13 will be produced that favor immunoglobulin-class switching of specific B cells to IgE (sensitization). Sensitization leads to the establishment of IgE^+ memory B cells and allergen-specific IgE antibodies. These are loaded by means of specific receptors (FCεRI, high affinity IgE receptor; FcεRII, low affinity IgE receptor) onto mast cells, basophils, monocytes, dendritic cells, and B cells. **b. Immediate reaction**. The crosslinking of IgE on mast cells by allergens leads to degranulation and the release of biologically active mediators (histamine, leukotrienes) and the immediate signs of allergy. **c. Late reaction**. Presentation of allergens to T cells, which become activated, proliferate and release proinflammatory cytokines (i.e. IL-4, -5, -13). This process may be enhanced by the IgE-mediated presentation of allergens to T cells. T_h2 cytokines (i.e. IL-5) induce tissue eosinophilia and the release of inflammatory mediators from eosinophils. **APC**-antigen presenting cell, **DC**-dendritic cell, **TCR**-T-cell receptor.

The understanding of equine hypersensitivity reactions remains in its infancy and the role T_h2 cells play in equine atopy is undetermined [2, 31]. Preliminary equine studies have reported the possibility of both T_h1 and T_h2 type T cells based on the detection of mRNA for IL-2 and IL-4 in cultured T cells [32]. A further study reported elevated IL-4 mRNA levels in the bronchoalveolar lavage (BAL) cells of horses with summer pasture-associated obstructive pulmonary disease suggesting a T_h2 cell involvement [31]. Another investigator demonstrated increased expression of the T_h2 cytokine profile (IL-4, IL-5) and decreased expression of the T_h1 profile (IFN-?) in RAO affected horses when compared to normal horses [33]. A different series of equine studies demonstrated increased numbers of T helper cells and IgE in BAL fluid collected from RAO affected horses that were exposed to natural antigens. This increase did not occur in BAL fluid of control horses [34, 35]. Additional information that implies IgE involvement in RAO is the fact that basophils from RAO affected horses are more responsive to environmental antigen-induced degranulation than normal horses further confirming the presence of antigen-specific IgE [31, 36]. Collectively, this information suggests the possible influence of the T_h2 cell and IgE in the pathogenesis of RAO.

Recurrent Airway Obstruction in the Horse

Recurrent airway obstruction (RAO) in the horse is believed to be a pulmonary hypersensitivity response to environmental antigens and affects the small airways of mature horses. Clinical signs of RAO vary and are dependent on the chronicity and severity of the disease. Horses with RAO may initially demonstrate signs of intermittent coughing that may progress to increased frequency of coughing, exercise intolerance, increased respiratory rate and effort, abnormal respiratory sounds, and weight loss as the disease process advances. These clinical signs arise from bronchospasm, hypersecretion of airway mucus, and migration of neutrophils and other inflammatory cells into the respiratory tract. Microscopically, the primary lesion in RAO affected horses is bronchiolitis characterized by diffuse epithelial hyperplasia, mucous plugging of airways, and the accumulation of mixed inflammatory cells such as lymphocytes and neutrophils [37].

RAO is a disease of domestication and is most common in the northern hemisphere where horses are stabled for a portion of the year and are fed hay that is cured in humid climates. Due to these circumstances, horses are confined to stalls for prolonged periods of time and are exposed to large amounts of antigens found in the environment and fodder. RAO results when susceptible horses develop a hypersensitivity to one or more of these antigens resulting in a pulmonary inflammatory response and subsequent airway obstruction. Although hundreds of antigens likely exist in the horse's environment, current evidence suggests thermophilic molds and actinomycetes that grow in damp hay as causative antigens [3]. Thermophilic molds and actinomycetes such as

Aspergillus fumigatus, *Faenia rectivirgula*, *Thermoactinomyces vulgaris*, and *Micropolyspora faeni* have been implicated based on various studies [38-40].

In a study by McGorum *et al*., the investigators found that RAO was induced by inhalation challenge with *M. faeni* and *A. fumigatus* in ROA affected horses thus implicating these antigens in the pathogenesis of RAO [39]. RAO was not noted in normal horses after inhalation challenge. Based on this information, the investigators suggested that RAO is a pulmonary hypersensitivity to specific antigens rather than a non-specific toxic response [39]. Another inhalation study found that aerosolized *M. faeni* caused changes in pulmonary function tests (respiratory rate, minute ventilation, pulmonary resistance, and Pa_{O2}) in RAO affected horses that were not noted in normal horses; thus implicated *M. faeni* as a possible causative antigen in the pathogenesis of RAO [38]. Although this evidence implicates specific antigens in the pathogenesis of RAO, challenge with individual antigens does not reproduce the severity of the natural occurring disease process. This suggests that RAO is a multifactorial disease with various antigens, duration of exposure, sensitivity of the individual horse, and other unidentified variables contributing to the pathogenesis of RAO.

Different types of inflammatory cells are found in the respiratory tract of horses with RAO and have been studied primarily through the evaluation of bronchoalveolar lavage fluid (BALF). Normal horses typically demonstrate alveolar macrophages and lymphocytes on routine BALF evaluation. BALF from RAO affected horses that are in remission demonstrate cell populations of primarily macrophages and lymphocytes and are similar to BALF from normal horses [34, 41]. In contrast, horses with clinical RAO

demonstrate an overall increase in the cellularity of the BALF with marked increase in the percentage of neutrophils present [42, 43].

The bronochoalveolar lavage (BAL) has also been used to further investigate the pathogenesis of RAO and the possibility of an immune mediated mechanism. In addition to the information mentioned in the previous section involving the presence of T_h1 and T_h2 cells in BALF of horses, the BAL has also been used to identify increased levels of histamine in ROA affected horses when compared to normal horses 5 hours after natural hay challenge [44]. In addition, Dirscherl and coworkers identified increased basophil responsiveness in 14 horses with RAO when compared to 8 control horses [36]. Other studies have demonstrated increased antigen-specific IgE and IgG antibodies to *M. faeni* and *A. fumigatus* in symptomatic and non-symptomatic RAO affected horses in comparison to normal horses [35, 45]. These findings of increased histamine levels, increased basophil responsiveness, and increased antigen-specific IgE in RAO affected horses suggests the involvement of an IgE mediated type I hypersensitivity in the pathogenesis of RAO.

In addition to the evaluation of IgE in BALF in the aforementioned studies, the investigators also evaluated antigen specific IgE in the serum of both RAO and normal horses. Quantification of systemic IgE is used as a diagnostic tool in the belief that atopic individuals have a higher level of circulating IgE when compared to non-atopic individuals. Although increased levels of antigen specific IgE were detected in the BALF in both studies, systemic IgE levels do not correlate with IgE levels in the BALF for any of the antigens evaluated (i.e. *M. faeni, A. fumigatus*) [35, 45]. Further, systemic IgE evaluation did not distinguish normal from RAO affected horses. This information

suggests that local and systemic IgE production may be regulated independent of one another and that the evaluation of serum IgE may have limited value in identifying specific causative antigens in equine RAO.

Clinical diagnosis of RAO is based on history, physical examination with special emphasis on respiratory auscultation, evaluation of arterial blood gas tension (i.e. Pa_{O2}), and BALF findings. Typically affected horses have a history of being housed in a stall for prolonged periods, may or may not have evidence of wheezes/crackles on thoracic auscultation, have low Pa_{O2}, and have neutrophil predominant cell populations on evaluation of BALF [3]. Other diagnostic modalities such as endoscopy of the upper airway, transtracheal tracheal wash, and thoracic radiography may help differentiate ROA from other diseases affecting the respiratory system.

Treatment of RAO is dependent on the severity and chronicity of clinical signs. Horses demonstrating early signs of RAO often respond to decreased amounts of antigen exposure. This is accomplished by providing environmental changes such as increased or complete housing outside and/or dietary changes (i.e. soaking hay in water, pelleted feeds). Horses with more severe/chronic clinical signs of RAO are often administered medications that modulate the inflammatory response such as aerosolized, orally, and/or parenterally administered corticosteroids. Other medications used to alleviate clinical signs include various bronchodilators, expectorants, and/or mucolytic agents [46, 47].

In addition to the previously noted diagnostic tests, the intradermal skin test (IDST) has been evaluated as a diagnostic modality in RAO. The IDST has been used in horses with the hope of identifying specific causative antigens involved in the pathogenesis of RAO. Once causative antigens are identified, select patients undergo

immunotherapy in an attempt to ameliorate clinical signs of RAO. The majority of IDST information has been extrapolated from human and small animal studies and a cloud of controversy surrounds its use and accuracy in identifying causative antigens in equine RAO.

Anatomy of the Equine Skin

The intradermal skin test utilizes the structure and cells of the skin as a reflection of how various other organs may respond to specific antigens. In addition, histologic examination of skin biopsy samples taken from intradermal skin test sites are often evaluated to identify the presence of inflammation and predominant cell types involved; from this information, one may be able to characterize the reaction (i.e. type I or IV hypersensitivity reaction) created by intradermal injection of a specific antigen. Another reason for histologic evaluation is to assess the safety of a particular intradermally injected antigen by evaluating the degree of trauma, tissue necrosis and/or edema created by intradermal injection of a particular substance at various concentrations. From this information, one may gain better insight on causative antigens in RAO as well as characterizing the type of inflammatory response, the cells involved, and the degree of trauma caused by intradermal injection of various substances at various concentrations. Because a section of this study evaluates skin biopsies microscopically, a brief review of the equine skin follows.

The horses' skin is comprised of the epidermis and the underlying dermis. The epidermis, with an average thickness of 0.053 mm, contains multiple layers of cells and is the outermost nonvascular layer of the skin [48, 49]. The cell layers of the epidermis

originate from a basal layer and are modified as they move superficially. The epidermis imparts many important functions of the skin such as pigmentation, immunologic regulation, and touch perception. The dermis is much thicker than the epidermis and serves to support the epidermis and provide flexibility to the skin through its composition of elastin and collagen. The thickness of the dermal layer varies throughout the horse depending on the region of the body and ranges from 1 to 6 mm (average 3.8 mm) [48, 49]. The thickest areas are found over the dorsum of the animal (head, mane, back, croup, tail) while the thinnest regions are found on the ventrum (udder, medial thigh, external genetalia) and the medial surfaces of the body and limbs [48-50]. The dermis also supports secondary structures such as hair follicles, sweat (apocrine) glands, sebaceous glands, and nerves (Figures 2 and 3).

Epidermis

The epidermis is the outermost layer of skin originating from the ectoderm and consists of multiple layers of cells that are continually shed and renewed. The epidermis is a stratified squamous epithelium that undergoes a pattern of proliferation, differentiation, and keratinization. The keratinization process starts at the germinal layer (stratum basale) where the cells undergo mitosis. As the cells migrate superficially through the stratum spinosum, granulosum, and corneum, they lose many of their cellular characteristics and act as a mechanical barrier as they reach the stratum corneum. During the process of keratinization, it is keratin, a highly stable fibrous protein, that is vital in providing the major barrier between the horse and the environment.

Several other unique cell types are dispersed throughout the epidermis. These cell types include Merkel cells, melanocytes, and Langerhans' cells. Merkel cells are located

in the basal region of the epidermis and function as slow-adapting touch mechanoreceptors [51-53]. Melanocytes are located in the basal layer of the epidermis, in the sweat gland ducts, in sebaceous glands, and in the outer root sheath and hair matrix of hair follicles. Melanin pigments produced by the melanocytes impart the coloration of skin and hair in animals. Skin and hair color is determined by the number, size distribution, and degree of melanization. Melanogenesis takes place in cytoplasmic granules called melanosomes through the actions of the enzyme tyrosinase. Melanin formation is controlled by genetics and melanocyte-stimulating hormone secreted by the pituitary gland [51]. Langerhans' cells are found throughout the epidermis as well as the dermal lymph vessels, lymph nodes, and dermis [53, 54]. Langerhans' cells originate from the bone marrow and are functionally and immunologically related to the monocyte-macrophage cell line. Langerhans' cells function as antigen-presenting cells to lymphocytes and act as initial receptors for cutaneous immune responses.

Dermis

The dermis originates from the mesoderm and is comprised of dense connective tissue that lies beneath the basement membrane and extends to the subcutis (hypodermis). The dermis consists of a lattice of dense irregular connective tissue along with collagen, elastin, and reticular fibers. Varoius cell types comprises the dermis but fibroblasts, mast cells, and macrophages predominate. Plasma cells, chromatophores, fat cells, and granulocytes are also found less frequently [55, 56]. The dermis is divided into a papillary layer and a reticular layer and in the horse, a third layer is found in certain regions of the body. The papillary layer is a thin layer consisting of loose connective tissue that conforms to the more superficial stratum basale of the epidermis. The reticular

layer comprises the bulk of the dermis and consists of thick densely arranged collagen and elastin fibers. The reticular layer also houses the hair follicles and sweat glands. Some sources refer to the papillary and reticular layers as the superficial and deep dermis, respectively. These terms are preferred due to the lack of rete ridges [51], an extremely irregular and complex architecture of the human dermo-epidermal junction, which is not typically found in horses [57, 58]. In the horse, an additional layer of connective tissue is present in the skin of the dorsal thorax, croup, dorsal surface of the back, lateral neck, and the skin present from the base of the tail to the tuber coxae. This unique layer, referred to as the cordovan-leather tissue layer, is found below the reticular layer and is composed of fine collagen, elastin, and reticular fibers [53, 59]. Smooth muscle fibers are found within the dermis in areas of the scrotum, teats, and penis while skeletal muscle is most notably found comprising the cutaneous trunci. Other areas of skeletal muscle are associated with the large sinus hairs of the facial region. Additional structures located within the dermis include blood vessels, lymph vessels, nerves, sebaceous glands, and sweat glands (Figure 4).

The subcutis or hypodermis is formed by a loose arrangement of collagen and elastic fibers and attaches the dermis to the deeper structures of bone and muscle. Within these fibers are variable amounts of fat cells that serve to provide energy, protect and support the body, and provide heat insulation to the body [51, 56].

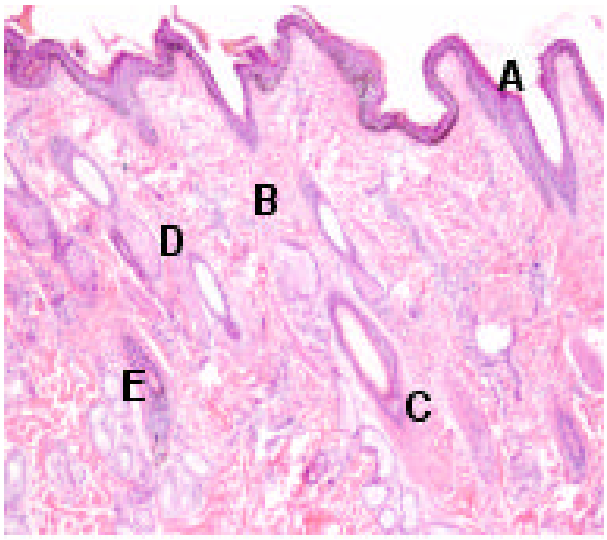

Figure 2:
Photomicrograph of the normal dermis and epidermis of the horse. A-epidermis, B-dermis, C-hair follicle, D-apocrine gland, E-sebacous gland. Notice the difference in thickness between the epidermis and dermis.
(H&E stain, Power 10x)

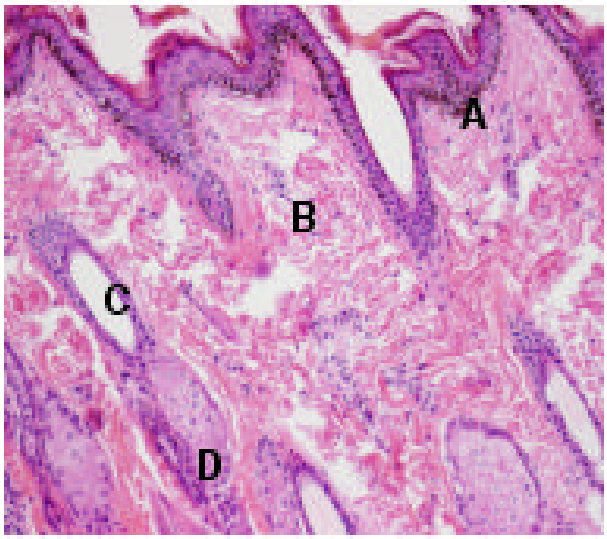

Figure 3:
Photomicrograph of the dermo-epidermal junction. A-epidermis, B-dermis, C-hair follicle, D-sweat gland.
(H&E stain, Power 20x)

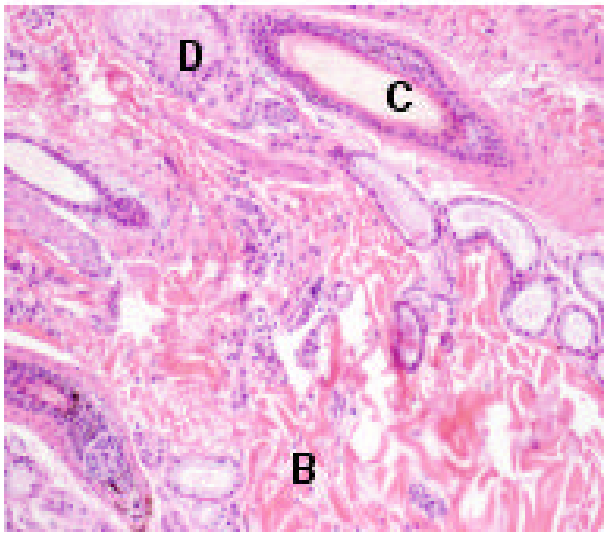

Figure 4:
Photomicrograph of the adnexal structures within the dermis. B-dermis, C-hair follicle, D-sweat gland.
(H&E stain, Power 30x)

Skin Response to the Intradermal Skin Test

The intradermal skin test (IDST) relies on the immunologic basis that specific injected antigens cause dermal mast cell degranulation and histamine release in individuals that are sensitized (i.e. have antigen specific IgE bound to mast cells) to a particular antigen. Degranulation of the mast cells leads to the grossly visible wheal, which is interpreted as a positive reaction to that specific antigen. Mast cells have both preformed mediators stored within granules and mediators that are synthesized at the time of activation. Preformed mediators include histamine, serotonin, tryptase, chymase, carboxypeptidase, TNF-α, eosinophil chemotactic factor, and neutrophil chemotactic factor [17, 60-62]. The wheal formed in the intradermal reaction is typically at its largest size 15-30 minutes post-injection and is primarily due to histamine induced smooth muscle contraction and increased vascular permeability leading to serum leakage and localized dermal edema [28, 61]. Other mediators that contribute, to a lesser degree, to wheal formation include preformed tryptase, chymase, and heparin [61]. Activation of mast cells also leads to the synthesis of other mediators including prostaglandins D2 and F2, thromboxane A2, leukotrienes B4, C4, D4, and E4 and platelet activating factor; some of these mediators are believed to be involved in the late phase reaction seen in IDST [55, 62-64]. Histologic evaluation of the immediate reaction created by mast cell degranulation is characterized by edema of the dermis and perivascular infiltration of neutrophils and eosinophils [28, 29].

Type I hypersensitivity reactions are similar to the immediate phase response as type I hypersensitivities are mediated by IgE and are primarily due to tissue mast cell degranulation. In allergic diseases that are associated with type I hypersensitivities, the

Fc portion of mast cells are sensitized and coated with antigen specific IgE. Upon repeated exposure to the same antigen, cross-linking of IgE occurs leading to mast cell degranulation that results in acute symptoms such as rhinitis, conjunctivitis, and asthma.

A dual-phase intradermal skin response involving a developing (4-12 hours) and late-phase reaction (>24 hours) may be observed subsequent to the immediate phase response during intradermal skin testing to particular antigens [28-30]. As the immediate hypersensitivity reaction begins to subside, various mediators released from mast cells such as eosinophil chemotactic factor, neutrophil chemotactic factor, IL-3, and IL-5 induce a localized inflammatory reaction with an influx of inflammatory cells [25]. The late-phase reaction is characterized clinically by dermal edema, erythema, induration and pruritus. On histologic exam of the late phase response, an influx of neutrophils, eosinophils and basophils occurs during the first six hours followed by infiltration of mononuclear cells (i.e. T cells) and fibrin deposition [29, 30, 65-70]. The late phase reaction may be a sequela to mast cell degranulation as mast cell mediators such as leukotrienes B4, C4, and D4, prostaglandin D2 and/or platelet activating factor have been detected in the skin within the time of the late phase reaction [71-73]; however eosinophils and T cells may also contribute [29, 74, 75]. This pattern of inflammatory cell infiltration closely resembles atopic dermatitis lesions suggesting that the late-phase reaction may be clinically more relevant to the naturally occurring disease than the immediate reaction [28].

The Intradermal Skin Test – Relationship to Respiratory Allergies

The IDST is intended to detect type I (IgE mediated) hypersensitivities. The concept of the cutaneous tissue serving as a mirror of local and systemic immune reactions is possible because antigen specific IgE is attached to mast cells found in the skin. However, sensitized mast cells are also found in many other areas of the body including the lung and gastrointestinal tract. Therefore, antigens that stimulate cutaneous mast cells may reflect an individual's sensitivity to specific antigens in other locations of the body such as mast cells found in the respiratory tract (i.e. respiratory allergies) and gastrointestinal tract (i.e. food allergies) [76].

Mast cells have been implicated in the pathogenesis of RAO in horses. In an equine study, the investigators compared lung mast cell numbers in normal horses and RAO affected horses and found more mast cells around airways in RAO affected horses when compared to normal horses [77, 78]. Another equine study evaluated the distribution of mast cells in the respiratory tract of normal horses and found mast cells at all levels of the respiratory tract with the greatest percentage found in the nasopharynx [79]. Mast cell structure was also evaluated and found to resemble human type I and type II mast cells [79]. In addition, investigators have found increased numbers of mast cells and increased levels of IgE in BAL fluid of RAO affected horses compared to normal horses [34, 35, 78]. Very little additional information is available in regards to the types and distribution of equine mast cells and most literature available on mast cell populations are in reference to the equine respiratory tract and how mast cells relate to the pathogenesis of RAO.

The Intradermal Skin Test in the Horse

To date, the intradermal skin test and serum enzyme-linked immunosorbent assay (ELISA) have been reported in the literature as diagnostic modalities utilized in an attempt to identify causative antigens in equine atopic diseases such as RAO, insect hypersensitivity, and recurrent urticaria [80-88]. There is, however, conflicting evidence concerning the use of the IDST and its reliability in defining causative antigens. In a study by McPherson *et al.*, the investigators performed intradermal skin testing with environmental antigens in 38 horses with RAO and 34 normal horses [40]. The investigators concluded that the intradermal skin test was an indicator of the cause of respiratory hypersensitivity based on the positive response to intradermal and inhalation challenge with *Micropolyspora faeni* in horses with RAO that was not noted in normal horses [40]. However, in the same study, poor positive correlation was found between intradermal skin testing and inhalation challenge in RAO affected horses and the use of another suspect antigen, *Aspergillus fumigatus*.

McGorum *et al*. performed inhalation challenges, on separate occasions, on 8 normal and 8 RAO affected horses using extracts of *Micropolyspora faeni*, *Thermactinomyces vulgaris*, and *Aspergillus fumigatus* [39]. The investigators found that RAO was induced in the RAO affected horses after challenge of *M. faeni* and *A. fumigatus*. RAO was not induced by any of the antigens after inhalation challenge in the normal horses, thus implicating *M. faeni* and *A. fumigatus* in the pathogenesis of RAO. In a follow-up study by the same investigators, intradermal skin testing with multiple concentrations of *M. faeni, T. vulgaris,* and *A. fumigatus* was performed on the same group of horses (8 normal and 8 RAO affected horses) 3-6 months after the first study

[89]. In this study, no significant differences in intradermal skin test reactions were noted between control and RAO affected horses with any of the antigens. In evaluation of the two studies, the investigators suggested that there is divergence of dermal and pulmonary reactivity to *M. faeni, T. vulgaris,* and *A. fumigatus* in the horse and that intradermal mold antigen testing is of limited value in the identification of causative antigens related to RAO in horses [89]. Interestingly, two aforementioned studies performed by Halliwell *et al*. and Schmallenback *et al*. have demonstrated increased levels of antigen specific IgE in BALF of ROA horses when compared to normal horses. However, an increase in IgE was not noted in serum samples of the RAO affected horses [35, 45]. This suggests that local and systemic production of IgE antibodies may be independent of one another and may offer another possible explanation why IDST has produced inconsistent results.

Further studies involving intradermal skin testing in horses have yielded equivocal if not conflicting results. A study performed by Beech and Gunson compared intradermal skin test results (25 antigens) between 25 normal horses and a non-specified number of horses with suspected allergic disease (chronic coughing, RAO, head-shakers). In this study the allergic horse group had a higher incidence of positive skin tests reactions when compared to the normal horses. However, no specific information was published involving the materials, methods, or results [80]. Evans and coworkers performed intradermal skin testing (58 allergens) on 6 horses with RAO, 8 horses with recurrent urticaria, and 11 clinically normal horses. Results from this study demonstrated positive skin test reactions in all three groups of horses with a significant difference in IDST reactions between normal and ROA horses for 3% of the allergens used. The same study also suggested that horses with RAO or urticaria had a greater total percentage of

allergen reactions than did clinically normal horses but the investigators were not able to make a distinction between the groups when evaluated individual allergens [85]. Results from the intradermal skin test study performed by Lowell involved 6 horses with RAO and 6 clinically normal horses. No difference in skin test reactions was noted between the two groups [88]. A study by Lorch *et al*. compared intradermal skin testing using 73 environmental allergens between 22 horses without atopy and 16 horses with RAO [84]. The investigators found that horses without atopy had a greater number of positive reactions (709 positive reactions) in the immediate and late-phase time periods when compared to horses with RAO (370 positive reactions). The investigators concluded that the IDST could not be used to diagnosis ROA in horses and the importance of positive skin test reactions should be carefully considered. A subsequent study performed by the same group of investigators found a greater proportion of horses with RAO, recurrent urticaria, and allergic dermatitis reacted to a greater number of IDST allergens at the 30 minute, 4 hour, and 24 hour time periods when compared to healthy horses [90]. In addition, the investigators stated that the diseased horses reacted to a significantly higher number of allergens when compared to normal horses. The results of these studies do not provide consistent and convincing evidence that the IDST can detect causative antigens in horses with RAO and/or other atopic conditions.

Justification for Study

Possible reasons for the disparity in intradermal skin test results in equine studies include variability of antigen selection and preparation, variability in antigen concentration, variability in intradermal injection technique, variability in time periods

when intradermal skin test wheals are evaluated post-injection, and the variability in the wheal response to intradermal injection of antigen in normal and atopic horses. Alternatively, the individual horse may demonstrate wheal variability because of differences in skin thickness, regions of the neck used for skin testing, and/or location of an injected antigen in relationship to another injected antigen.

The majority of information and techniques used in intradermal skin testing in horses has been extrapolated from human and small animal studies without accurate validation in horses. This study is necessary to validate the IDST in horses and to evaluate the IDST as a potential diagnostic tool in the identification of causative antigens in equine RAO. Validation will be provided by evaluating the variance in wheal response to intradermal injection of positive control substances in an individual horse and between a group of horses. If significant wheal variability is noted within and/or between several horses to known positive controls (i.e. histamine), future use of the IDST in horses may be futile. This study will also establish appropriate concentrations of specific intradermal skin test control substances and antigens for the horse. In addition, we will attempt to differentiate intradermal skin tests responses between normal and RAO affected horses. Further, this study will characterize the cellular response created by intradermal injections of control substances and test antigens in normal and ROA affected horses. This information may then serve as a reference point for additional intradermal skin testing with causative antigens.

Chapter 2:

Variability in the wheals produced by intradermal injection of three stimulants in the horse.

Introduction

Allergic diseases are frequently encountered problems in equine medicine. Two commonly reported allergic diseases in the horse are recurrent airway obstruction (RAO) and insect hypersensitivity. Multiple studies suggest that RAO results from hypersensitivity to environmental antigens such as molds, dust, and fungal spores while insect hypersensitivity results from reactions to insect bites from *Culicoides* species [3, 39, 91]. Interestingly, definitive diagnosis of causative antigens and treatment of these diseases has been difficult despite the frequency of which they are noted in clinical practice. Diagnostic tests used to define specific causative antigens in equine allergies include serologic tests such as the enzyme-linked immunosorbant assay (ELISA) and dermal tests such as the intradermal skin test (IDST). However, most of the information and techniques used in intradermal skin testing in horses has been extrapolated from human and small animal studies without accurate validation in horses.

The IDST gains its usefulness as a diagnostic modality in RAO and insect hypersensitivity by using the cutaneous tissue as a mirror of local and systemic immune reactions. This principle is possible because antigen specific IgE antibodies are attached to mast cells found in both the skin and in many other areas of the body including the lung and gastrointestinal tract. Therefore, antigens that stimulate cutaneous mast cells may represent antigens that stimulate other mast cells found in various organs besides the

skin [76]. Specific intradermally injected antigens cause mast cell degranulation in sensitized individuals. Degranulation of mast cells subsequently leads to the release of preformed mediators such as histamine and the generation of other mediators such as leukotrienes. These mediators lead to the formation of a grossly visible wheal, which is interpreted as a positive reaction to that specific antigen. The wheal formed in the intradermal reaction is primarily due to histamine induced vascular permeability and secondary edema; this reaction is typically at its largest size 15-30 minutes post-injection [61].

The intradermal skin test is an accepted method of antigen detection in humans and small animals. However, a paucity of studies in equine medicine have evaluated the intradermal skin test and its potential use in the diagnosis of allergic diseases. Results of these studies have been inconsistent casting uncertainty on the viability of the IDST as a diagnostic test in equine allergic disease [80, 82, 84, 85, 88]. It is our contention that before IDST can be applied as a clinical diagnostic tool in equine medicine, validation of the consistency of the IDST must be established (i.e. will the intradermal injection of 0.0005 mg of histamine produce a similarly sized wheal within one horse and among several horses). Validation may begin by providing information about the variability of wheal reactions to known positive controls (i.e. histamine) and by evaluating the hypothesis that there is minimal variation in wheal diameter created by intradermal skin test control substances within a single horse or between a group of horses.

Histamine is the most commonly used positive control substance in human and small animal intradermal skin testing. Histamine has also been used as a mainstay in equine skin testing. Compound 48/80 and Phaseolus vulgaris (PHA) have also been

investigated as potential positive control stimulants [92-96]. When injected intradermally, histamine leads to an immediate non-specific inflammatory response and the formation of a visible wheal. Compound 48/80 has been studied in both canine and equine skin testing and leads to wheal formation via mast cell degranulation [92, 96, 97]. PHA is a plant lectin that leads to a delayed type-IV hypersensitivy reaction via its mitogenic properties [93, 95, 98].

The objectives of this study were to evaluate the variance in wheal diameter created by three intradermal stimulants, histamine, compound 48/80, and PHA, and to histologically evaluate the cellular response created by the intradermal injection of these stimulants. Specifically, the objectives were to: 1) evaluate the variance in wheal diameters created by repeated intradermal injections of the three stimulants at 5 concentrations within a horse, 2) evaluate the variance in wheal diameters created by repeated intradermal injections of the three stimulants at 5 concentrations between 12 horses, 3) establish at what time points the wheal responses to each stimulant are best evaluated, and 4) histologically evaluate skin biopsy samples from the wheals created by intradermal injection of each stimulant at three different times. Through this information, the intradermal skin test can by validated or refuted as a consistent diagnostic test in the evaluation of equine allergic diseases. This information may then serve as a reference point for additional intradermal skin testing with causative antigens by validating or refuting the repeatability/consistency of the IDST in horses.

Materials and Methods

Animals:

Twelve adult horses ranging in age from 4-24 years old (9 mares, 3 geldings) were selected for the study based on the absence of historical and clinical evidence of allergic disease. Breeds represented included Thoroughbred (n=6), Arabian (3), Quarter Horse (1), Spanish Barb (1) and Saddlebred (1). All horses were examined within one week of the study and assessed as clinically normal based on physical exam, complete blood count, and serum biochemistry profile. Prior to participation in the study, all horses were maintained on pasture as part of the university herd. Each horse was vaccinated annually with equine rhinopneumonitis, eastern/western equine encephalitis, tetanus, and rabies and dewormed with ivermectin every 12 weeks. The study protocol was approved by the Virginia Tech Animal Care and Use Committee.

Housing and Treatment:

Horses were housed separately in a research facility one day prior to intradermal skin testing. Intradermal skin testing commenced on day 2 of the study and the horses returned to their normal environment on day 3. A daily physical exam was performed on each horse for the 3 days that were spent at the research facility. The horses were housed in a 10 x 10 stall with free access to an individual walkout paddock. The horses were fed 2 flakes of grass hay twice daily along with free access to fresh water.

Stimulant Preparation and Intradermal Skin Testing

Three intradermal stimulants, histamine [a], compound 48/80 [b], and PHA [c] were used in the study. A preliminary experiment was conducted to determine the optimal concentrations of the three stimulants prior to the initiation of this study. Five concentrations of each stimulant were prepared for this study. Histamine concentrations included 0.005, 0.001, 0.0002, 0.0004, and 0 mg/ml. The concentrations of compound 48/80 included 50, 37.5, 25, 12.5, and 0 µg/ml. PHA concentrations included 1, 0.7, 0.4, 0.1, 0 mg/ml. The 0 (control) concentration consisted of phosphate-buffered saline (PBS) diluent used to prepare the stimulants (Table 1). Each stimulant (0.1 ml) at each concentration was drawn up into tuberculin syringes (27 gauge, 3/8 inch needle). A total of 30 tuberculin syringes were prepared for IDST (5 concentrations x 2 repeats of each concentration x 3 stimulants = 30) for each side of each horse. Both sides of the neck were utilized for IDST; therefore 60 syringes were prepared per horse. Each group of 30 stimulants was placed in a random order in a syringe tray as determined by a computer generated randomization chart. Each horse was assigned a different randomization chart but the same random order was used for both sides of the neck on an individual horse.

In addition, 0.1 ml of each stimulant at their respective highest concentration (histamine at 0.005 mg/ml, compound 48/80 at 50 µg/ml, PHA at 1 mg/ml) was prepared for intradermal injection and subsequent biopsy collection. Two syringes of each stimulant (3 stimulants x 2 repeats = 6 total syringes) were prepared for each horse.

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[a] **Histamine,** Sigma-Aldrich, St. Louis, MO
[b] **Compound 48/80**, Greer Labs, Lenoir, NC
[c] **Phaseolus vulgaris,** Sigma-Aldrich, St. Louis, MO

Table 1 – Intradermal Skin Test Stimulants and Concentrations

Histamine (mg/ml)	0.005	0.001	0.0002	0.0004	0
48/80 (μg/ml)	50	37.5	25	12.5	0
PHA (mg/ml)	1	0.7	0.4	0.1	0

* 2 repeats of each concentration on each side of the neck = 10 injections of each stimulant/side of the neck or 30 total injections/side

A 15-inch x 8-inch rectangle was clipped (Oster No. 40 blade) on both sides of the lateral aspect of the neck of each horse. A grid was drawn with a permanent marker within the clipped area on each side of the neck consisting of 10 squares across and 3 squares down. Each horse was then sedated with xylazine [d] (0.5mg/kg, IV). Intradermal skin test injections (30/side; 60 total/horse) were performed on both sides of the neck of each horse simultaneously by 2 clinicians. The stimulants used for the biopsy samples (0.5 and 4 hour) were injected intradermally outside of the skin test grid and labeled accordingly. The same 2 clinicians performed all intradermal injections on all 12 horses and were blinded to the contents of each syringe. Measurements of the wheals produced by each stimulant on each horse were made at 0.5-hours, 4-hours, and 24-hours post-injection by one blinded evaluator.

Skin punch biopsies (6 mm) were collected at the highest concentration for all three stimulants at 0.5-hours, 4-hours, and 24 hours. For the 0.5 and 4-hour skin biopsies, the samples were taken from the intradermal injections outside the test grid. For the 24- hour skin biopsies, the highest concentration of histamine, compound 48/80, and PHA was identified within the test grid (after the 24 hour measurement had been recorded) and subsequently taken from within the test grid. This was done to reduce the number of total intradermal injections. Lidocaine 2% (1 ml) was injected subcutaneously and a 6 mm skin punch biopsy device was used to collect the samples. The biopsy site was closed with a simple interrupted suture. The samples were preserved in 70% ethanol for a minimum of 24 hours. The samples were then cut in half, routinely fixed in paraffin blocks, cut at a thickness of 5 µm with a rotary microtome, and stained with hemotoxylin and eosin (H&E). The biopsy sections were subjectively evaluated microscopically for

[d] **Xylazine**, Fermenta Animal Health Co., Kansas City, MO

the presence of inflammation, predominate cellular infiltrate, and presence of edema. A subjective scale was followed by a pathologist who examined all slides. The grading system for inflammation and edema was: 0-none, 1-mild, 2-modest, 3-moderate, and 4-severe.

Statistical Analysis for Histamine and Compound 48/80

Concentration response curves were made for both stimulants. Based on evaluation of the raw data, measurable wheal responses for histamine and compound 48/80 were detected at the 0.5-hour time. Simple linear models were used to represent the relationship between concentration of stimulant and response (wheal diameter) for histamine and compound 48/80. The simple linear models for histamine were put on a log scale because of the wide range of histamine concentrations used. The log scale provided a better representation of the concentration response curve. The mean wheal diameter, Y-intercept and slope were calculated for histamine and compound 48/80 at the 0.5-hour time.

In addition, the variance in wheal diameter for both stimulants at the 0.5-hour time was evaluated within a horse and between horses. A mixed-model ANOVA was used to estimate the variance components within a single horse and between 12 horses. The mixed procedure of the SAS system [e] was used for calculating the ANOVA. Evaluation of what time points (0.5, 4, 24 hours) that produced consistent wheals for each stimulant was performed by examination of the raw data.

[e] **Sas System,** version 8.01 Sas Institute Inc. Cary, NC 27513

Results for Histamine and Compound 48/80

The complete blood counts and serum biochemistry profiles did not reveal any significant abnormalities in any of the horses prior to initiation of the study. Daily physical exams were within normal limits. All horses tolerated intradermal injection of histamine and compound 48/80 with only minor discomfort observed during the procedure. No significant or severe reactions were noted to any of the injections. All control (PBS) injections produced 9-11 mm wheals that were gone by the 4-hour reading.

Consistent wheal measurements were obtained at the 0.5-hour time for histamine. All concentrations of histamine produced dose-related wheals ranging from 9-22 mm across all concentrations at the 0.5-hour time. However, only the higher concentrations of histamine (0.001, 0.005 mg/ml) produced wheals at the 4-hour time. No wheals were noted at the 24-hour time regardless of concentration. Therefore the 4- and 24-hour wheal readings were discarded for histamine due to the lack of a consistent response at these times.

A simple linear model was used to represent histamine (Figure 1). The Y-intercept at the 0.5-hour time, representing the wheal diameter when the concentration of histamine was 0 mg/ml (i.e. control), was 10.12 mm. The slope of the line [b] demonstrated an increase of 1156 mm in wheal diameter for every one (1) unit increase of histamine (Table 2). The maximum concentration in this experiment was 0.02 mg/ml; therefore the maximum wheal diameter was approximately 23 mm.

Compound 48/80 produced wheals ranging from 9-15 mm at the 0.5-hour time. Four of 12 horses demonstrated wheal reactions at the 4-hour time at the highest concentration (50 µg/ml). No wheals were noted at the 4-hour time for all other

concentrations and no wheals were noted at the 24-hour time regardless of concentration. Therefore the 4- and 24-hour wheal readings were discarded for compound 48/80 due to the lack of a consistent response at these times.

Compound 48/80 was also expressed as a simple linear model (Figure 2). The Y-intercept at the 0.5-hour time, representing the wheal diameter when the concentration of compound 48/80 was 0 µg/ml (i.e. control), was 9.574 mm. The slope of the line [b] demonstrated an increase of 0.015 mm for every one (1) unit increase of compound 48/80 (Table 2). However, compound 48/80 did not demonstrate a clinically significant concentration response (i.e. 100 unit increase in 48/80 would cause a 1.5 mm increase in wheal diameter).

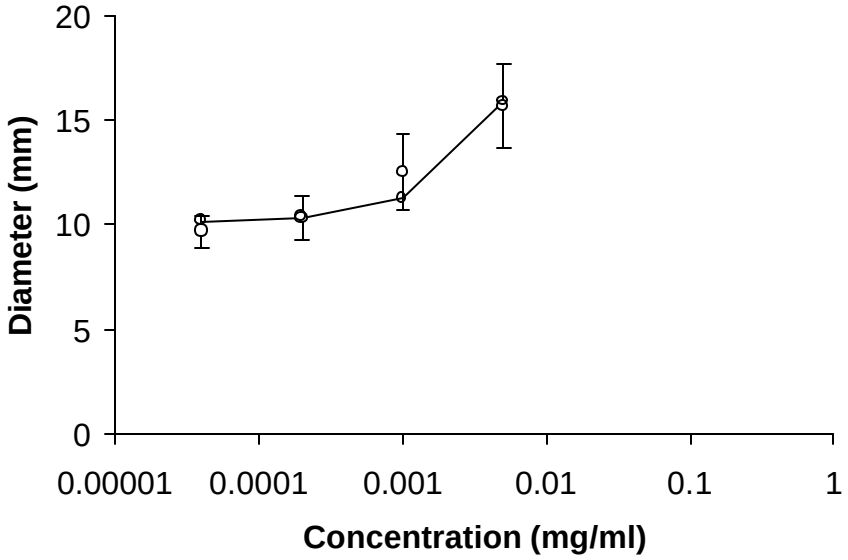


Figure 1: Model of Histamine (0.5-hour). Linear model of the mean wheal diameters (open circles) of 12 horses (total = 48 wheals/concentration) 0.5 hours after intradermal injection of 5 concentrations of histamine. The line represents the fitted model for histamine at 0.5-hours across 5 concentrations. Model is on a logarithmic scale (note- wheals created at 0 concentration not plotted because of logarithmic scale)

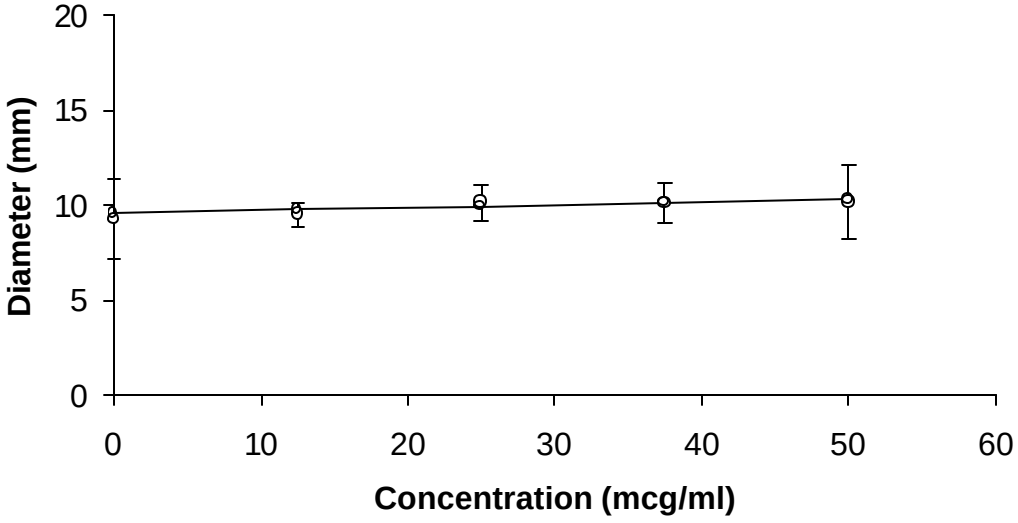


Figure 2: Model of Compound 48/80 (0.5-hour). Linear model of the mean wheal diameters (open circles) of 12 horses (total = 48 wheals) 0.5 hours after intradermal injection of 5 concentrations of compound 48/80. The line represents the fitted model for compound 48/80 at 0.5 hours across 5 concentrations.

Table 2: Mean, Standard Deviation and 95% Confidence Interval for Y-intercept and Slope of Histamine and Compound 48/80 at the 0.5 hour time. The Y-intercept represents the mean of all wheals (total 48 wheals) created by the intradermal injection of histamine or compound 48/80 at the 0 (control) concentration at the 0.5 hour time in 12 horses. The slope represents the rate at which the wheal size increases across increasing concentrations of histamine or compound 48/80. STD-standard deviation.

	Histamine (0.5 hour)	**Compound 48/80** (0.5 hour)
Variable A (Y-intercept)		
Mean	10.127	9.575
STD	0.526	0.266
95% Confidence Interval		
Upper Limit	11.157	10.097
Lower Limit	9.096	9.053
Variable B (Slope)		
Mean	1156.911	0.015
STD	266.189	0.017
95% Confidence Interval		
Upper Limit	1678.641	0.049
Lower Limit	635.181	-0.018

* 95% Confidence Interval = The population mean will be included in the confidence interval in 95% of the samples; 5% of the samples will be outside the confidence interval

The calculated variance components for histamine reveled a wheal to wheal variation within a horse of 1.3993 and a horse to horse variation of 0.3592 at the 0.5-hour time. The calculated variance components for compound 48/80 revealed a wheal to wheal variance of 0.7451 and a horse to horse variation of 0.1343 at the 0.5-hour time (Table 3).

Table 3: **Wheal to wheal variation within and between horses for Histamine and Compound 48/80.** Number represents the amount of wheal to wheal variation in millimeters within a horse and amount of horse to horse variation in millimeters at the 0.5 hour time.

	Histamine	Compound 48/80
	0.5 hrs	0.5hrs
Wheal to wheal variation within a horse (standard error)	1.399 (0.1481)	0.745 (0.07967)
Horse to horse variation (standard error)	0.359 (0.2067)	0.134 (0.07914)

Histologic Examination of Histamine and Compound 48/80

Based on examination of the raw data and the pattern of wheal formation, the biopsy samples of histamine (0.005 mg/ml) were examined microscopically at 0.5-hours (Table 4). Three samples taken from three different horses were randomly selected for evaluation at 0.5 hours. These samples demonstrated modest to severe superficial and deep edema, vascular margination of neutrophils and eosinophils, and minimal exudation of neutrophils into the interstitium (Figures 3-6).

Based on examination of the raw data and the pattern of wheal formation, the biopsy samples of compound 48/80 (50 µg/ml) were examined microscopically at 0.5-hours (Table 4). Three samples taken from three different horses were randomly selected for evaluation at 0.5 hours. These samples demonstrated mild to modest superficial and deep edema and rare perivascular lymphocytes (Figures 7-8).

Table 4: Histologic evaluation of biopsy sites 0.5 hours after intradermal injection of histamine or compound 48/80 grading edema and inflammation. Grading scale: 0-none, 1-mild, 2-modest, 3-moderate, 4-severe, NA-not applicable. The character of inflammation is noted were applicable; MO-macrophage, LO-lymphocyte, NO-neutrophil, EO-eosinophil.

Stimulant	Time (hrs)	Edema	Inflammation	Character of Inflammation
Histamine	0.5	3	0	No Cells
Histamine	0.5	4	0	No Cells
Histamine	0.5	2	0	No Cells
48/80	0.5	2	0	No Cells
48/80	0.5	1	0	No Cells
48/80	0.5	2	0	No Cells

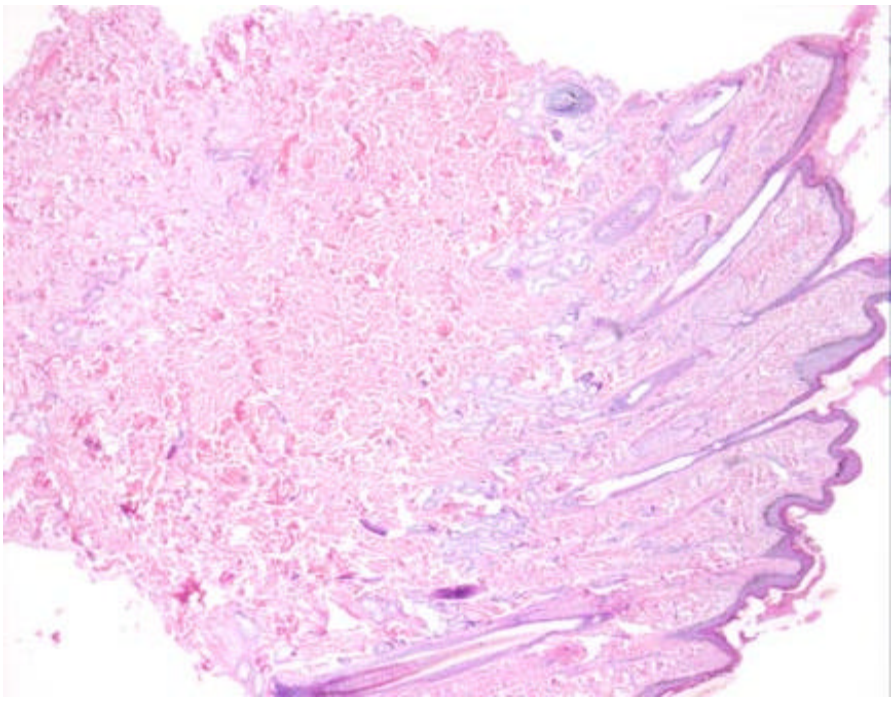


Figure 3: Photomicrograph of a skin biopsy 0.5 hours after intradermal injection with histamine (0.005 mg/ml) demonstrating edema of the superficial and deep dermis. H&E stain; Magnification – 4x.

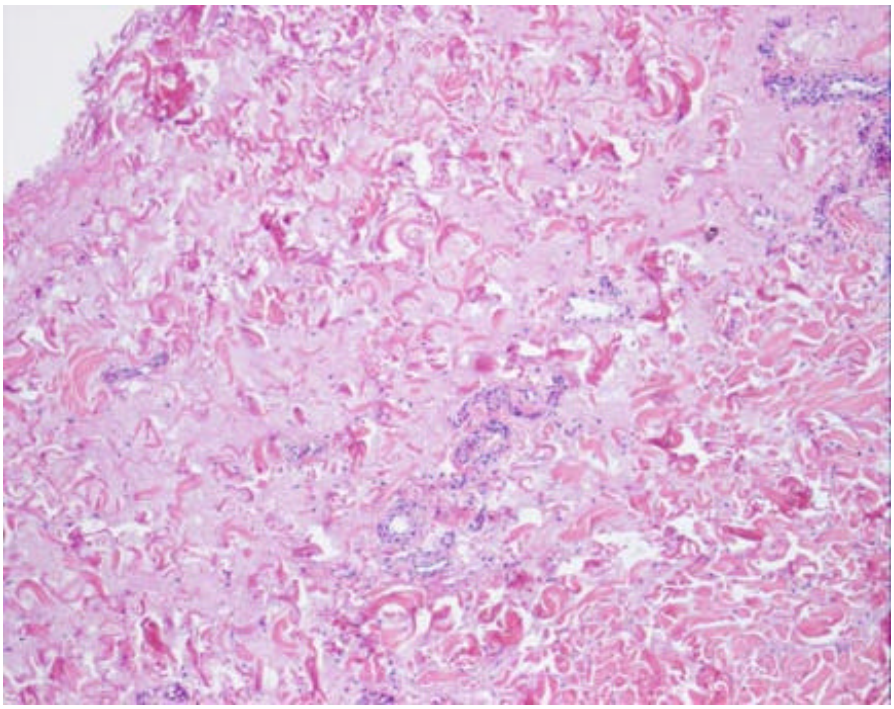


Figure 4: Photomicrograph of a skin biopsy 0.5 hours after intradermal injection with histamine (0.005 mg/ml) demonstrating edema of the deep dermis. H&E stain; Magnification – 10 Power.

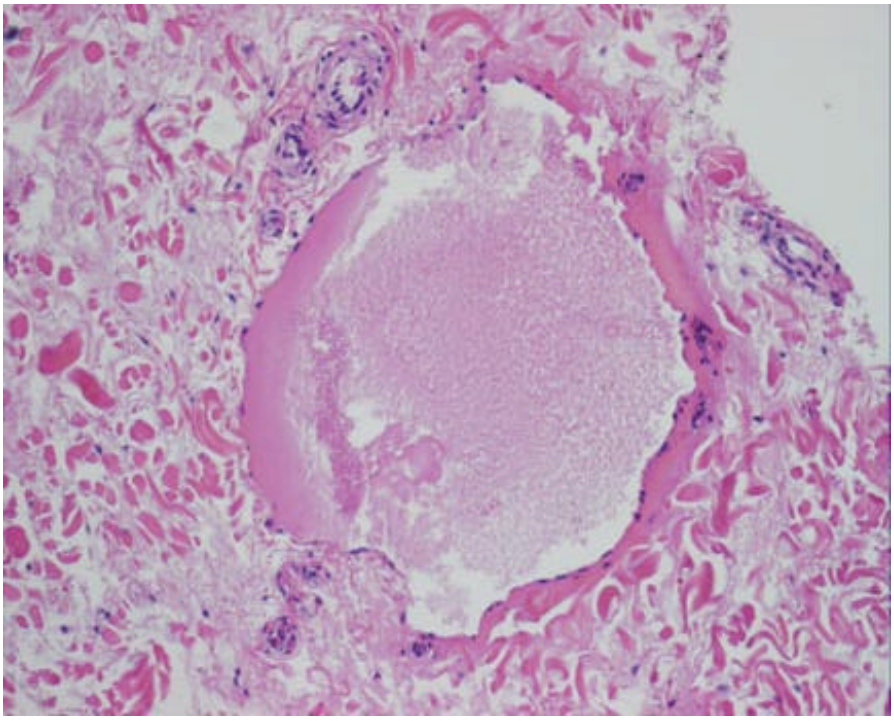


Figure 5: Photomicrograph of a biopsy site 0.5 hours after intradermal injection with histamine (0.005 mg/ml) demonstrating proteinacious fluid within a lymphatic. H&E stain; Magnification – 20x.

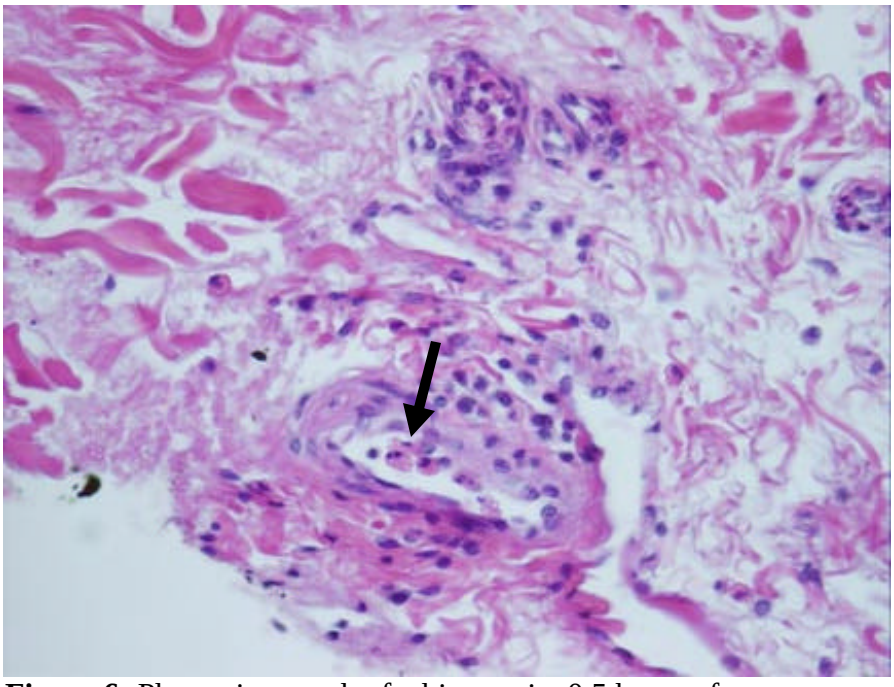


Figure 6: Photomicrograph of a biopsy site 0.5 hours after intradermal injection with histamine (0.005 mg/ml) demonstrating vascular margination of neutrophils and eosinophils (arrow). H&E stain; Magnification – 40 Power.

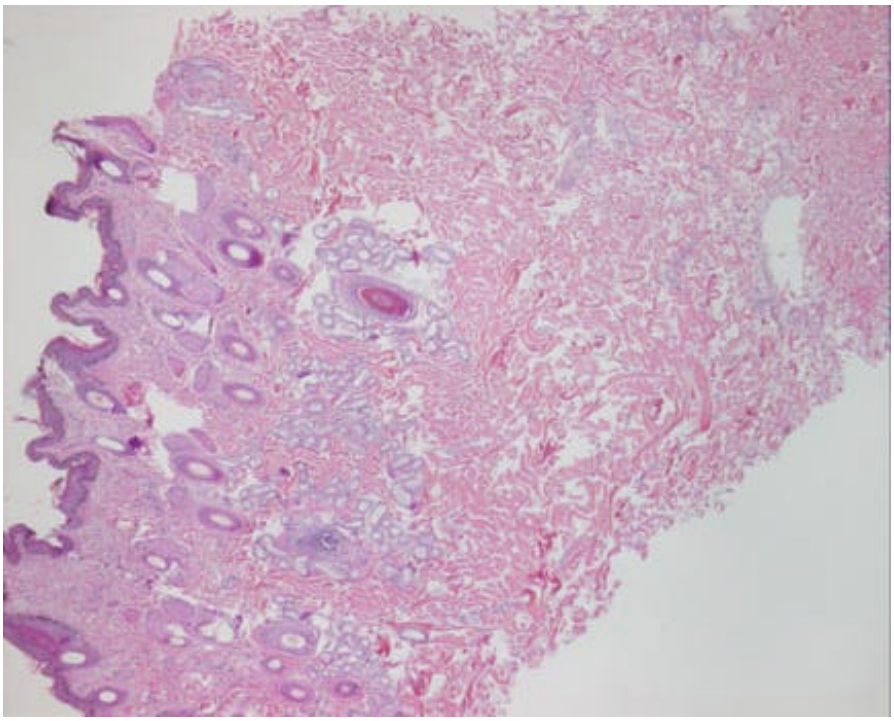


Figure 7: Photomicrograph of a skin biopsy 0.5 hours after intradermal injection of compound 48/80 (50 µg/ml) demonstrating dermal edema. H&E stain; Magnification – 4x.

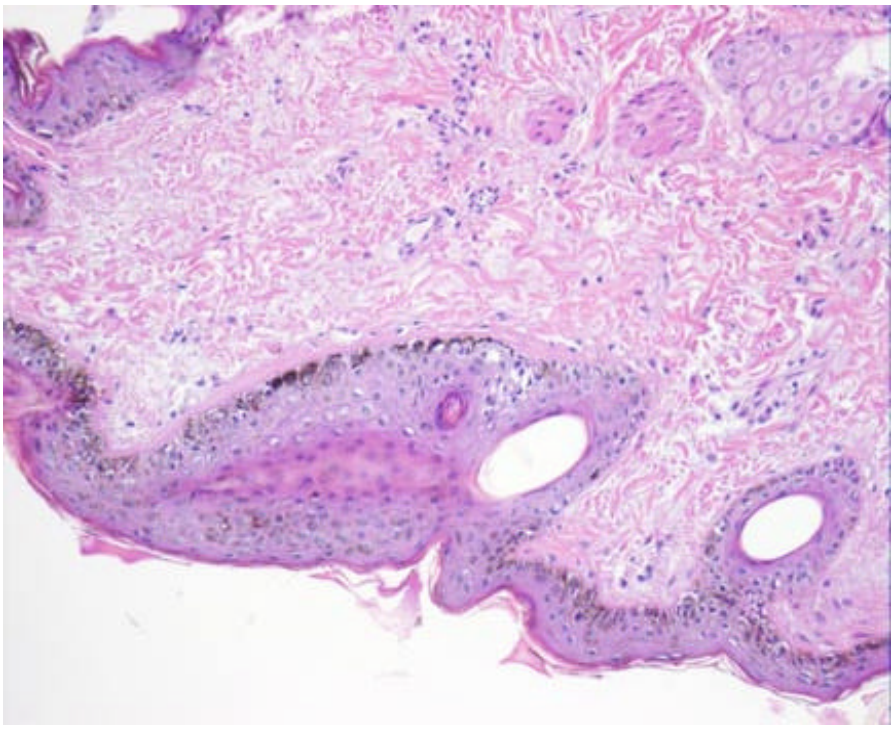


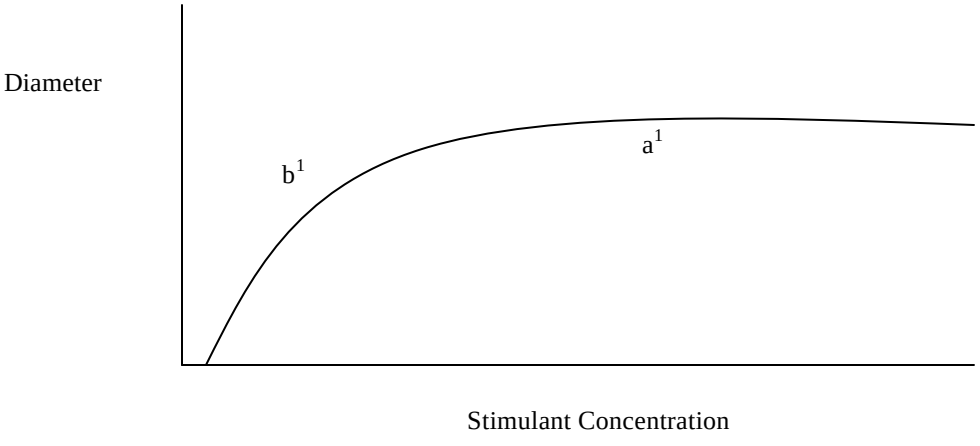
Figure 8: Photomicrograph of a biopsy site 0.5 hours after intradermal injection of compound 48/80 (50 µg/ml) demonstrating edema of the superficial dermis. H&E stain; Magnification – 20x.

Statistical Analysis for PHA

Concentration response curves were made for PHA. Based on evaluation of the raw data, measurable wheal responses for PHA were at the 4- and 24-hour times. The data for PHA did not fit a simple linear model; therefore the Chapman-Richard's Equation [I] was used to express PHA. The relationships between stimulant concentrations and wheal diameters were modeled to evaluate the rate of rise [b] and the maximum wheal diameter (plateau [a]) (Figure 3).

In addition, the variance in wheal diameter of PHA at the 4- and 24-hour times was evaluated within a horse and between horses. A mixed-model ANOVA was used to estimate the variance components within a single horse and between 12 horses. The mixed procedure of the SAS system was used for calculating the ANOVA. Evaluation of what time points for each stimulant could be consistently used was performed by examination of the raw data.

Figure 9: Diagrammatic representation of stimulant concentration verse diameter graphs for PHA in normal horses using the Chapman-Richard's equation (a^1=plateau, b^1=rate of rise).



[I] Chapman-Richard's Equation: $Y = a[1-e^{-bX}]^c$

Results for PHA

All horses tolerated intradermal injection of PHA with only minor discomfort observed during the procedure. No severe reactions were noted to any of the injections although the horses did demonstrate pain upon digital palpation of the wheals produced by PHA at the 4- and 24-hour times. Several of the horses demonstrated large wheal formation (50-75 mm) to the highest concentration of PHA (1 µg/ml) at the 24-hour time. All control (PBS) injections produced 9-11 mm wheals at the 0.5-hour time that were gone by the 4-hour time. PHA produced consistent wheal readings at the 4- and 24-hour time periods. At the 0.5-hour time, dose related wheals were produced ranging from 8 to 18 mm. This included the control (0) concentration. The 0.5-hour wheal readings were discarded due to equivocal results at this time (i.e. wheals produced by all concentrations of PHA ≅ wheals produced by negative control). PHA produced dose-related wheals ranging from 16 to 48 mm at the 4-hour time. At 24-hours, dose-related wheals were produced ranging from 11 to 75 mm. The majority of the horses demonstrated a greater wheal size at the 24-hour time when compared to the 4-hour time independent of stimulant concentration.

The plateau at 4 hrs, representing the maximum wheal diameter across concentrations, was 25.804 mm. The rate of rise [b] demonstrated an increase of 9.065 mm for every one (1) unit increase of PHA at the 4-hour time (Table 4). The plateau at 24 hrs, representing the maximum wheal diameter across concentrations, was 39.938 mm. The rate of rise [b] demonstrated an increase of 5.827 mm for every one (1) unit increase of PHA at the 24-hour time (Table 4).

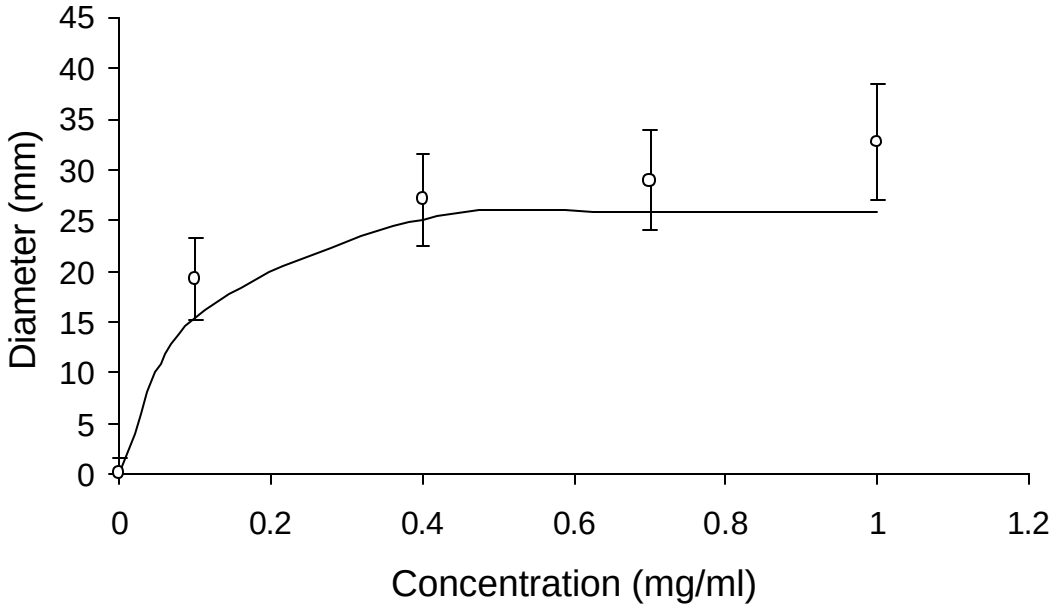


Figure 10: Model of Phaseolus vulgaris (4-hours). Non-linear model of the rate of rise and maximum mean wheal diameters (open circles) of 12 horses (total = 48 wheals/concentration) 4 hours after intradermal injection of 5 concentrations of PHA. The line represents the fitted model for PHA at 4 hours across 5 concentrations.

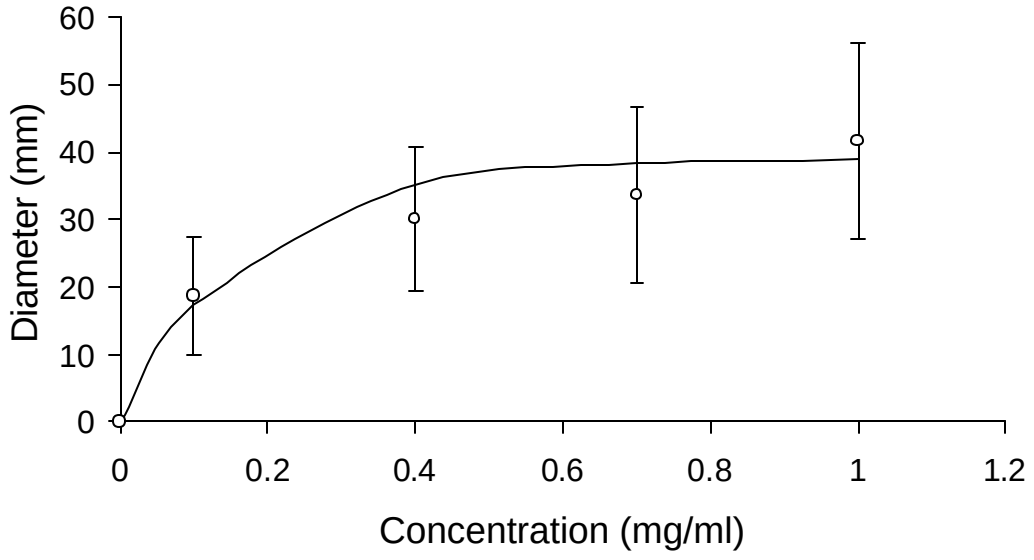


Figure 11: Model of Phaseolus vulgaris (24-hours). Non-linear model of the rate of rise and maximum mean wheal diameters (open circle) of 12 horses (total = 48 wheals/concentration) 24 hours after intradermal injection of 5 concentrations of PHA at 24 hours. The line represents the fitted model for PHA at 24 hours across 5 concentrations.

Table 5: Mean, Standard Deviation and 95% Confidence Interval for Plateau and Rate of Rise for PHA at the 4-and 24-hour times. The plateau (A) represents the mean of the maximum wheal diameters created by the intradermal injection of PHA across 5 concentrations of PHA at the 4- and 24-hour time in 12 horses. The rate or rise (B) represents the rate at which the wheal size increases across increasing concentrations of PHA. STD-standard deviation.

	PHA (4 hours)	**PHA** (24 hours)
Variable A (Plateau)		
Mean	25.804	38.938
STD	5.402	12.527
95% Confidence Interval		
Upper Limit	36.393	63.491
Lower Limit	15.215	14.386
Variable B (Rate of Rise)		
Mean	9.065	5.827
STD	1.989	2.224
95% Confidence Interval		
Upper Limit	12.963	10.187
Lower Limit	5.167	1.467

* 95% Confidence Interval = The population mean will be included in the confidence interval in 95% of the samples; 5% of the samples will be outside the confidence interval.

The calculated variance components for PHA reveled a wheal to wheal variation within a horse of 6.76 and a horse to horse variation of 10.108 at the 4-hour time. At the 24-hour time, the calculated variance components for PHA revealed a wheal to wheal variance of within a horse of 43.445 and a horse to horse variation of 41.977 (Table 6).

Table 6: Wheal to wheal variation within and between horses for PHA. Number represents amount of wheal to wheal variation in millimeters within a horse and amount of horse to horse variation in millimeters at the 4- and 24-hour time.

	Phaseoulus vulgaris (PHA)	
	4 hrs	24 hrs
Wheal to wheal variation within a horse (standard error)	6.760 (0.713)	43.450 (4.720)
Horse to horse variation (standard error)	10.108 (4.753)	41.977 (21.858)

Histologic Exam

Based on examination of the raw data and the pattern of wheal formation, the biopsy samples of PHA were examined microscopically at 4- and 24-hours (Table 7). Three samples from different horses were randomly selected for evaluation at each time (4 and 24 hours). At 4 hours, the samples demonstrated severe deep and superficial edema and hemorrhage, lymphatic ectasia with proteinaceous fluid, and moderate perivascular exudation of neutrophils with extension into the interstitium and peri-adnexal structures (Figures 12-13). At 24 hours, the samples demonstrated moderate to severe superficial and deep edema with decreased evidence of hemorrhage, lymphatic ectasia with proteinaceous fluid, and a mixed inflammatory response characterized by moderate to severe perivascular exudation of mononuclear (lymphocytes/macrophages) cells with extension into the interstitium and peri-adnexal structures (Figures 14-16). Fewer numbers of perivascular neutrophils were noted with some extension into the interstitium and peri-adnexal structures.

Table 7: **Histologic evaluation of biopsy sites 4 and 24 hours after intradermal injection of PHA grading edema and inflammation**. Grading scale: 0-none, 1-mild, 2-modest, 3-moderate, 4-severe, NA-not applicable. The character of inflammation is noted were applicable; MO-macrophage, LO-lymphocyte, NO-neutrophil.

Stimulant	Time (hrs)	Edema	Inflammation	Character of Inflammation
PHA	4	4	3	Neutrophilic
PHA	4	4	3	Neutrophilic
PHA	4	4	3	Neutrophilic
PHA	24	4	3	Mixed; MO, LO, NO
PHA	24	3	3	Mixed; MO, LO, NO
PHA	24	3	4	Mixed; MO, LO, NO

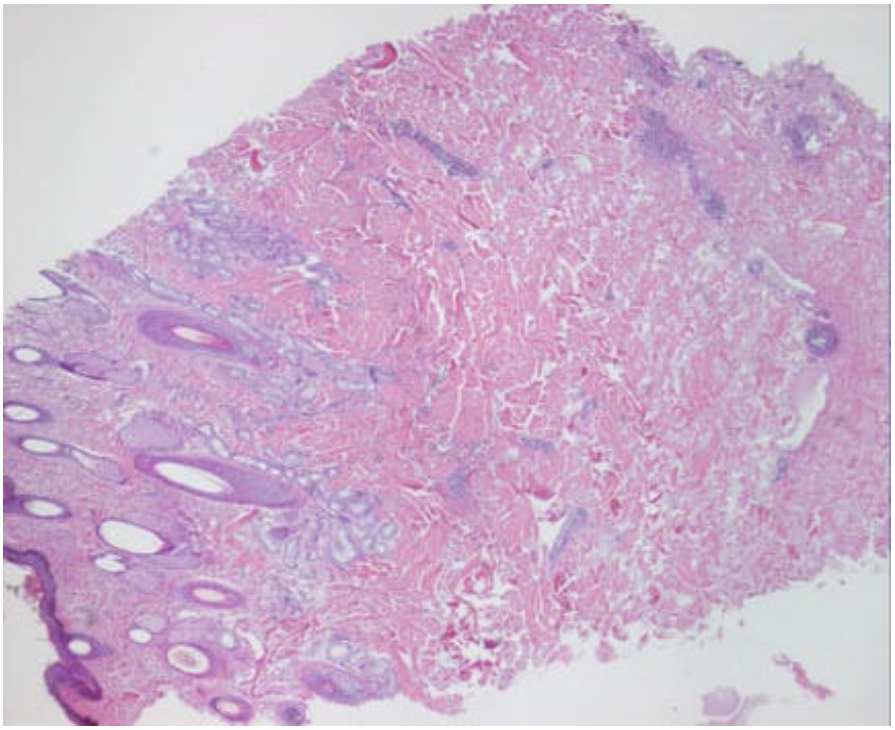


Figure 12: Photomicrograph of a biopsy site 4 hours after intradermal injection of PHA (1 mg/ml) demonstrating dermal edema. H&E stain; Magnification – 4x.

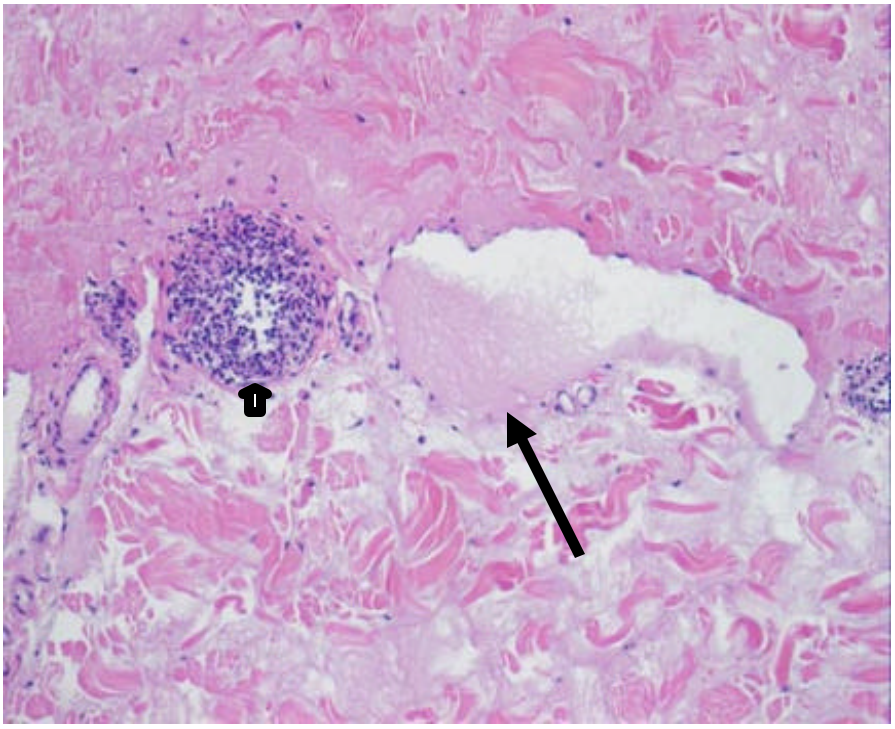


Figure 13: Photomicrograph of a biopsy site 4 hours after intradermal injection of PHA (1 mg/ml) demonstrating dermal edema, proteinacious fluid within a lymphatic (arrow) and perivascular exudation of neutrophils (arrowhead). H&E stain; 20x.

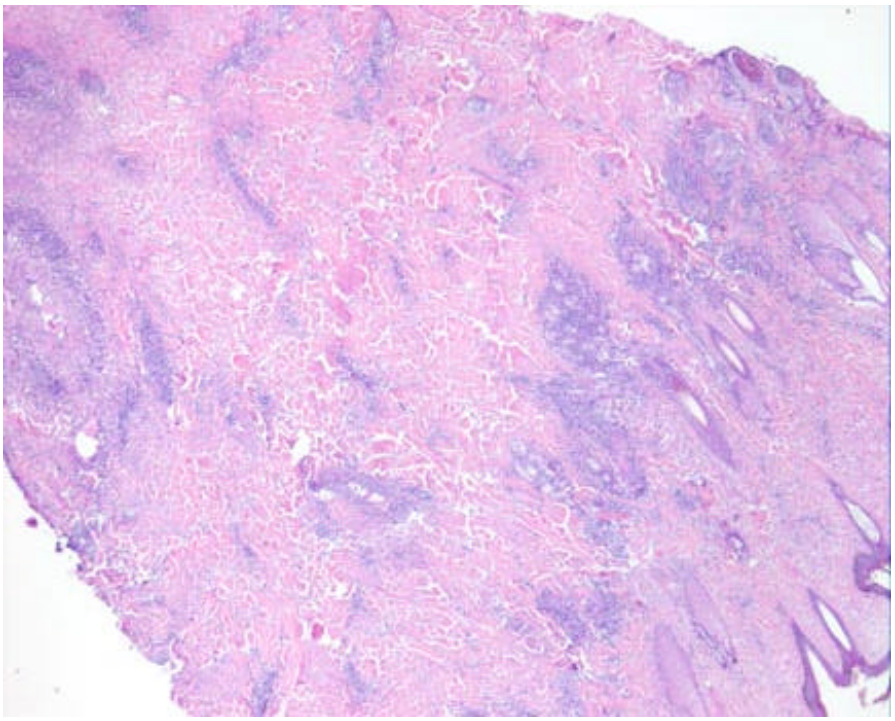


Figure 14: Photomicrograph of a biopsy site 24 hours after intradermal injection of PHA (1 mg/ml) demonstrating marked cellular inflammation of the dermis. H&E stain; Magnification–4x.

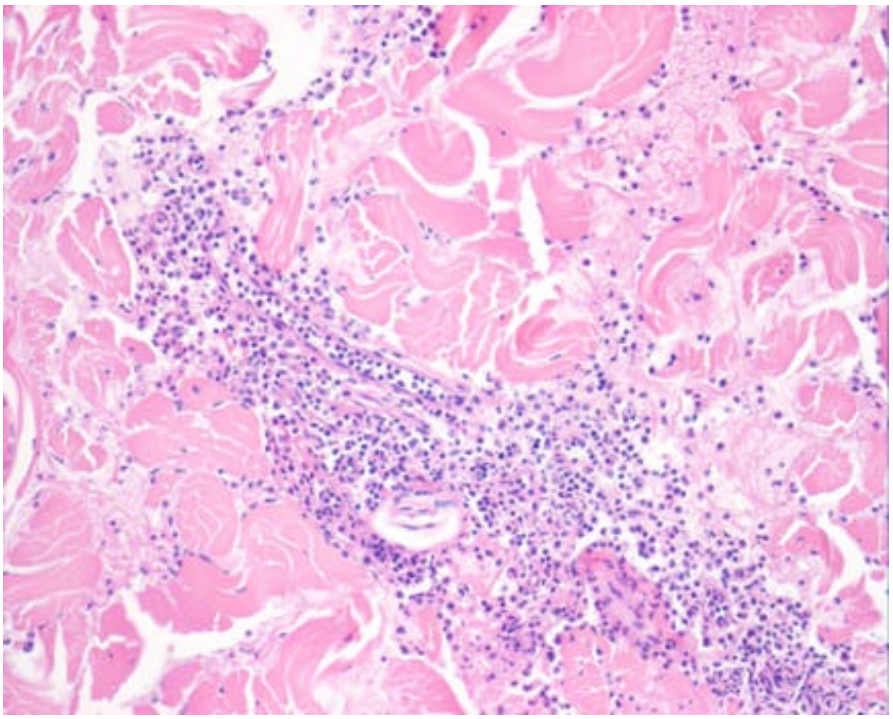


Figure 15: Photomicrograph of a biopsy site 24 hours after intradermal injection of PHA (1 mg/ml) demonstrating margination and extravasation of neutrophils and macrophages in the deep dermis. H&E Stain; Magnification – 20x.

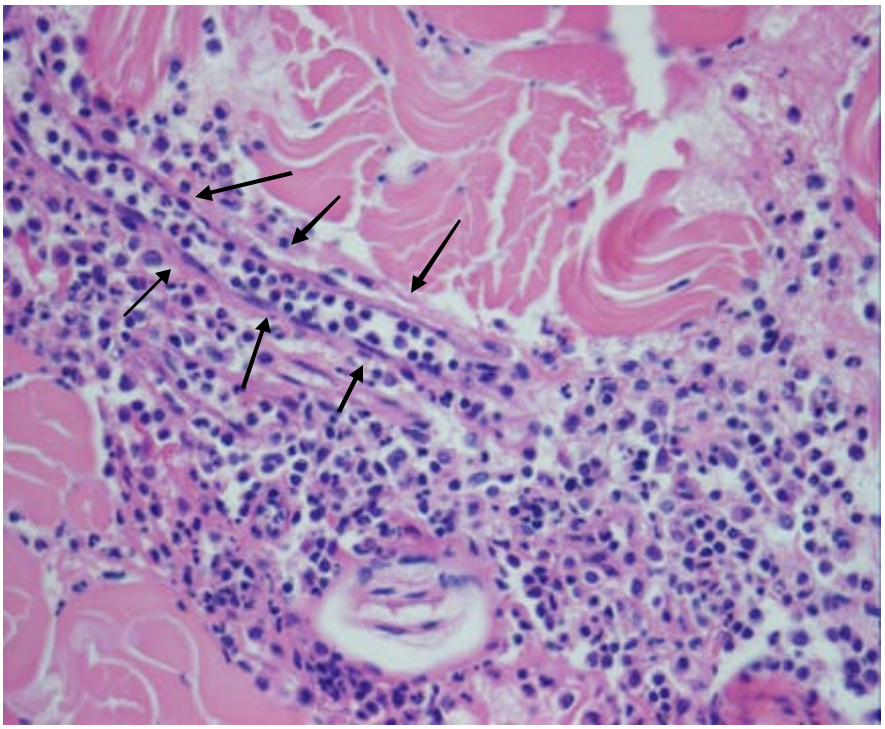


Figure 16: Photomicrograph of a biopsy site 24 hours after intradermal injection of PHA (1 mg/ml) demonstrating margination and extravasation of neutrophils and macrophages. Arrows outline a blood vessel in long axis. H&E Stain; Magnification – 40 x. [picture 10]

Control (PBS) biopsy samples were also examined microscopically at 0.5, 4, and 24 hours. The 0.5 and 24 hour time periods were normal and demonstrated no changes. However, at the 4 hour time, mild edema was noted with rare perivascular neutrophils within the deep dermal vessels along with poteinaceous fluid within lymphatics.

Discussion

Although the use of the intradermal skin test in the diagnosis of equine allergic diseases such as RAO and insect hypersensitivity is a common practice, we are unaware of any controlled studies that have critically evaluated the reliability/repeatability of the IDST. Results of intradermal skin testing in horses with RAO and horses without atopic conditions have been contradictory [80-82, 84, 85]. This suggests that various factors such as antigen selection, antigen concentration, intradermal skin testing procedures, and horse variability can contribute to the inconsistency noted in IDST. In the study reported here, there was minimal wheal to wheal variability within a horse and minimal variability between horses for histamine and compound 48/80 at the 0.5-hour time and PHA at the 4-hour time. However, PHA at the 24-hour time demonstrated a significant amount of wheal variability both within individual horses as well as between 12 horses.

Histamine is the most widely used and reliable positive control for intradermal skin testing. Intradermal injection of histamine results in a visible wheal and reflects the non-specific inflammatory response capability of the skin [96]. Histamine is a vasoactive amine that causes contraction of vascular endothelial cells and release of vascular smooth muscle relaxants. When histamine is released from mast cells or injected intradermally, it results in leakage of plasma into surrounding tissues and wheal formation. This was confirmed by histologic examination of histamine biopsy samples collected at 0.5 hours that demonstrated modest to severe superficial and deep dermal edema. Intradermal histamine induced wheal formation reflects vascular responsiveness (vasodilation) of skin and occurs independent of the presence of mast cells [92]. Therefore, histamine does not measure the ability of the mast cell to degranulate in response to antigen stimulation.

This study suggests that histamine produces consistent wheals within and between horses in response to intradermal injection at the 0.5-hour time based on the minimal wheal variance and the concentration response curve noted.

Compound 48/80 has been investigated as a positive control for intradermal skin testing in various species including humans, dogs, and horses [96, 97, 99]. Compound 48/80 is a mast cell secretagogue that produces a similar wheal response to intraderamally injected histamine [92, 99]. Compound 48/80 is a synthetic polyamine that interacts with mast cell membrane G proteins, activates phospholipase C, increases intracellular calcium stores and activates protein kinase C [97, 99, 100]; These events culminate in mast cell degranulation [92, 100]. Compound 48/80 may have an advantage over histamine as a positive control for intradermal skin testing because it induces mast cell activation and degranulation and reflects both mast cell responsiveness and vascular reactivity in the skin [92, 99, 100]. This sequence of events closely mimics the response to intradermally injected test antigens making compound 48/80 an ideal positive control. Mast cell degranulation imitates the natural course of events seen in type-I hypersensitivities in which high affinity IgE receptors (FceRI) on mast cells are cross-linked by specific antigens leading to mast cell degranulation. Mast cells subsequently release preformed mediators such as histamine, serotonin, tryptase, chymase, TNF-α, eosinophil chemotactic factor, and neutrophil chemotactic factor [17, 60-62]. When activated, mast cells also synthesize additional mediators such as prostaglandins and leukotrienes. Release of preformed and synthesized mediators leads to increased vascular permeability, edema, and migration of inflammatory cells representing the typical immediate type I hypersensitivity responses.

In a study involving compound 48/80 as a positive control for IDST in dogs, the investigators concluded that compound 48/80 was an unreliable positive control for skin testing in normal dogs because 2 of 11 dogs were non-responsive to compound 48/80. However, the investigators suggest that compound 48/80 may be a reliable positive control in atopic dogs [92]. In the only equine study involving compound 48/80, the investigators concluded that at 30 minutes post-injection, compound 48/80 at a concentration of 1000 µg/ml resulted in a reaction comparable to histamine in horses without skin disease and an inflammatory cell pattern similar to that noted in type-I hypersensitivity reactions [96]. However, the compound 48/80 wheal was less intense and took longer to develop when compared to histamine. This may have been due to the gradual release of histamine induced by compound 48/80 or a lower concentration of inflammatory mediators released in response to compound 48/80 than intradermal injection of histamine [96]. The investigators also provided evidence to support the hypothesis of compound 48/80's mast cell degranulation properties by demonstrating higher numbers of mast cells at the dermo-epidermal junction in untreated and saline-treated sites when compared to compound 48/80 treated sites. However, this was only detected in 1 of 3 horses.

In this study, compound 48/80 did not result in an appreciable concentration response curve with the highest concentration (50 µg/ml) producing a similar size wheal as the negative (PBS) control in all horses. Similar to histamine, only the 0.5-hour time provided complete and consistent results after evaluation of the raw data. Both the 4- and 24-hour times had essentially no measurable wheals regardless of the concentration of compound 48/80 administered. Wheal size ranged from 9-15 mm at the 0.5-hour time

regardless of the concentration of compound 48/80. A dose response may have not been observed because too low of a dose was used. The aforementioned equine study demonstrated wheal response to compound 48/80 with a concentration of 1000 µg/ml [96]. Canine studies have used concentrations ranging from 0.05 µg/ml to 1000 µg/ml [92, 97] while human studies involving the use of compound 48/80 have utilized concentrations ranging from 10 µg/ml to 35 mg/ml [65, 67, 99].

The ideal concentration of compound 48/80 has not been identified in horses. The information from this study suggests that 50 µg/ml of compound 48/80 does not produce a reliable positive control wheal. This concurs with the previously mentioned equine study in which concentrations of 0.01, 0.1, 1, 10, and 100 µg/ml of compound 48/80 did not result in significant wheal formation [96]. In this study, the wheals produced by the highest concentration of histamine were of larger size (mean=15.67 mm) than the wheals produced by the highest concentration of compound 48/80 (mean=10.14 mm). The negative control (PBS) in this study created wheals at the 0.5-hour time that ranged in size between 9 and 11 mm. Therefore, it is difficult to state that the wheals created by compound 48/80 were the result of mast cell degranulation or by the simple presence of an intradermally injected substance. The previous equine study did evaluate the cellular characteristics of the compound 48/80 (1000 µg/ml) sites histologically at 0.5 hours and found an inflammatory cell pattern involving increased numbers of perivascular neutrophils and eosinophils when compared to controls. Microscopic examination also demonstrated decreased numbers of mast cells that was interpreted as increased mast cell degranulation at compound 48/80 test sites. These histologic findings were similar to that noted in type-I hypersensitivity. In this study, histologic exam of compound 48/80 (50

µg/ml) at 0.5-hours revealed mild to modest superficial and deep edema. However, no significant inflammatory infiltrate was noted and specific stains were not used to identify mast cells.

Based on the information from this study, it is difficult to make conclusive statements on the usefulness of compound 48/80 as a positive control in IDST. Higher concentrations of compound 48/80 may produce different results. However, at the concentrations used in this study, intradermal injection of compound 48/80 yielded minimal variance in wheal size within and between horses.

PHA was used in this study to evaluate the late phase reaction and T cell function. In contrast to histamine and compound 48/80, PHA involves a delayed type IV response and is mediated by T lymphocytes. PHA is in the family of lectins; of special importance is their ability to act as mitogens (i.e. stimulation of lymphocyte growth and cell division). Mitogens such as PHA and Concanavalin A specifically stimulate thymus derived T cells [93]. In a study investigating the use of PHA for the detection of combined immunodeficiency (CID) foals, the investigators found that PHA does cause a delayed type-IV reaction that is best recorded at 24 hours post injection [93].

PHA was chosen for this study to evaluate the late-phase reaction that is sometimes reported in atopic conditions and intradermal skin testing [28-30, 101]. This late-phase reaction is caused by the activation of allergen-specific T cells after hours to days of the immediate hypersensitivity reaction [25]. The late-phase reaction is characterized by a strong T-cell infiltration and eosinophil activation in humans [28, 30, 68, 70, 101]. It is speculated that allergen-specific IgE may have a role in activating allergen-specific T cells by facilitating antigen presentation [25]. This pattern of

inflammatory cell infiltration closely resembles atopic dermatitis lesions suggesting that the late-phase reaction may be clinically more relevant to the naturally occurring disease than the immediate reaction. In this study, PHA produced dose-related wheals at the 4- and 24-hour time periods. However, a significant amount of variance was noted at the 24-hour time that may represent the individual horse variation in T-cell response at the latest evaluation time (24 hours). The information from this study suggests that PHA has minimal variance at 4 hours and stimulates a type IV response based on histologic exam of PHA biopsies that demonstrated significant perivascular infiltration of mononuclear cells with extension into the interstitium at 24 hours. In addition, histologic exam also demonstrated severe edema and hemorrhage as a result of intradermal injection of PHA. This was evidenced clinically as pain on digital palpation of the PHA sites.

Conclusions

In this study, minimal variability was noted in the immediate, 0.5-hour, wheal reactions for histamine and compound 48/80. Minimal variability existed within multiple injections of the same concentration of the same stimulant within individual horses as well as between 12 horses. Histamine caused a predictable concentration response curve with the size of the wheal increasing as concentration increased. Upon evaluation of the raw data, only the 0.5-hour time provided complete and consistent histamine results at the concentrations used in this study. The 4-hour time yielded sporadic measurements while at the 24-hour time there were no measurable wheals involving any horse at any concentration of histamine. Like histamine, compound 48/80 produced consistent results at the 0.5-hour time. However the wheals produced by compound 48/80 at the highest concentration were of similar size to those produced by the negative control. Therefore, injection of compound 48/80 at the concentrations used in this study may have caused wheal reaction due to the simple presence of an intraderamlly injected substance rather than mast cell degranulation. Minimal variation was noted with 48/80 but a dose-related wheal response was not noted.

PHA caused a significant dose response curve with maximal wheal size recorded at the 24-hour time. PHA also had the greatest variance in wheal diameter at the 24-hour time both within individual horses as well as between 12 horses. Although it is believed that allergies are of the type-I hypersensitivity (IgE mediated), PHA may be considered as an additional positive control to evaluate T cell activity, the late-phase response and delayed type-IV hypersensitivities.

We conclude that intradermal injections of histamine and compound 48/80 produce statistically consistent and repeatable wheals when injected intradermally within individual horses as well as between 12 horses at the 0.5-hour time. Intradermal injection of PHA produced statistically consistent and repeatable wheals when injected intradermally within individual horses as well as between 12 horses at the 4-hour time period. However, the 24-hour time for PHA had significant variability within individual horses as well as between 12 horses.

This information can be applied to the intradermal skin test in which multiple antigens are injected intradermally in an attempt to identify causative antigens in allergic diseases. We can therefore conclude that intradermal injections of positive controls such as histamine, compound 48/80, and PHA, will provide a consistent and repeatable wheal at specific times and therefore place more credibility in the wheal responses created by positive antigens.

CHAPTER 3:

Comparison of Intradermal Skin Test Results Between Normal and ROA affected horses.

Introduction

Recurrent airway obstruction (RAO) is a common respiratory disease affecting the horse and is characterized by intermittent periods of airway obstruction evidenced clinically by wheezing, coughing, exercise intolerance, tachypnea, and increased respiratory effort [3]. RAO has no breed or sex predilection and commonly affects middle age to older horses (>7 years). RAO is reported to be a hypersensitivity disease that occurs when horses are exposed to various antigens such as *Micropolyspora faeni*, *Aspergillus fumigatus*, and *Thermoactinomyces vulgaris* [3, 39, 40]. Diagnosis of RAO is based on clinical signs and evaluation of bronchoalveolar lavage (BAL) fluid. Although these diagnostic tests may confirm the presence of RAO, they do not provide evidence to the causative antigens involved in airway hypersensitivity. Research has been conducted to better understand the pathophysiology and treatment of RAO. However, most current treatment modalities revolve around the palliation of clinical signs rather than addressing the underlying disease process.

The intradermal skin test has been established as a diagnostic tool in the human and small animal atopic patients and allows more precise identification of causative antigens in allergic patients. Once causative antigens are identified, selected patients are treated with hyposensitization therapy in an attempt to ameliorate the frequency and severity of clinical signs. The intradermal skin test has also been used in equine patients

to identify causative antigens in atopic diseases such as RAO, but results have been inconsistent (as discussed in chapter 1).

The purpose of this study was to 1) compare wheal reactions between normal horses and horses with RAO using the three known positive control stimulants, histamine, compound 48/80, and Phaseolus vulgaris (PHA), along with the environmental antigen *Aspergillus* and 2) compare, histologically, the cellular response created by the intradermal injection of *Aspergillus* between normal horses and horses with RAO. Specifically, we hope to evaluate the hypothesis that RAO affected horses react differently than normal horses to various intradermally injected stimulants. This may be accomplished by identifying differences in the concentration response curves and/or maximum wheal diameters between normal horses and RAO affected horses using positive control stimulants and an environmental antigen. In addition, we will compare histologically the cellular characteristics of skin biopsy samples from the wheals created by intradermal injection of *Aspergillus* between normal and RAO affected horses.

Materials and Methods

Animals:

Normal Horses:

Five normal adult horses ranging in age from 6-24 years old (5 mares) were selected for the study based on the absence of historical and clinical evidence of allergic disease. All horses were Thoroughbreds that were examined within one week of the study and assessed as clinically normal based on history and physical exam. Prior to participation in the study, all horses were maintained on pasture as part of the university herd. Each horse was vaccinated annually with equine rhinopneumonitis, eastern/western encephalitis, tetanus, and rabies and dewormed with ivermectin every 12 weeks.

Horses with RAO:

Five adult RAO affected horses ranging in age from 6-23 years old (2 mares, 3 geldings) were selected for the study based on historical and clinical evidence of RAO. RAO affected horses were donated to the Virginia-Maryland Regional College of Veterinary Medicine for a chronic history of RAO. Breeds represented included Thoroughbred (n=2), Tennessee Walking Horse (1), Paint (1), and Appaloosa (1). RAO was confirmed weeks to months prior to commencement of this study based on the results of clinical exam, complete blood count, serum biochemistry profile, arterial blood gas analysis, bronchoalveolar lavage, and challenge with moldy alfalfa hay. Prior to participation in the study, all horses were maintained on pasture as part of the university herd. Each horse was vaccinated annually with equine rhinopneumonitis, eastern/western encephalitis, tetanus, and rabies and dewormed with ivermectin every 12 weeks. At the

time of this study, all RAO affected horses were in remission and did not demonstrate signs of respiratory compromise. The study was approved by the Virginia Tech Animal Care and Use Committee.

Housing and Treatment:

Horses were housed separately in a research facility one day prior to intradermal skin testing. Intradermal skin testing commenced on day 2 of the study and the horses returned to their normal environment on day 3. A physical exam was performed on each horse for the 3 days that were spent at the research facility. The horses were housed in a 10 x 10 stall with free access to an individual walk out paddock. The horses were fed 2 flakes of grass hay twice daily along with free access to fresh water.

Stimulant Preparation and Intradermal Skin Testing

Three positive control intradermal stimulants, histamine [a], compound 48/80 [b], and PHA [c], were used along with one environmental allergen, *Aspergillus* [d] mix in the study. Five concentrations of histamine (0.02, 0.004, 0.0008, 0.00016, 0 mg/ml), PHA (0.5, 0.2, 0.08, 0.032, 0 mg/ml), and *Aspergillus (*4000, 2000, 1000, 500, 0 PNU/ml) were prepared along with 3 concentrations of compound 48/80 (50, 25, 0 µg/ml). Phosphate buffered saline (PBS) was used as the 0 (control) concentration and the diluent to prepare the stimulants (Table 1). Each stimulant (0.1 ml) at each concentration was drawn up into tuberculin syringes (27 gauge, 3/8inch needle). A total of 18 tuberculin syringes were prepared for IDST (5 concentrations x 3 stimulants + 3 concentration x 1 stimulant = 18) for each side of each horse. Both sides of the neck were utilized for IDST; therefore 36

syringes were prepared per horse. Each group of 18 stimulants was placed in a random order in a syringe tray as determined by a computer generated randomization chart. Each horse was assigned a different randomization charts but the same random order was used for both sides of the neck on each horse. In addition, 0.1ml of *Aspergillus* mix at the highest concentration (4000 PNU/ml) was prepared for intradermal injection and subsequent biopsy collection; 2 syringes/horse of *Aspergillus* mix were prepared for this purpose.

A 15-inch x 8-inch rectangle was clipped (Oster No. 40 blade) on both sides of the lateral aspect of the neck of each horse. A grid was drawn with a permanent marker within the clipped area on each side of the neck consisting of 10 squares across and 2 squares down. Each horse was then sedated with xylazine [e] (0.5mg/kg, IV). Intradermal skin test injections (18/side) were performed on both sides of the neck of each horse simultaneously by 2 clinicians. The additional intradermal *Aspergillus* mix (4000 PNU/ml) injections intended for biopsy were administered outside the skin test grid and labeled accordingly. The same 2 clinicians performed all intradermal injections on all 10 horses and were blinded to the contents of each syringe. Measurements of the wheals formed by each stimulant on each horse were made at 0.5 hours, 4 hours, and 24 hours post-injection by the same blinded evaluator.

Skin punch biopsies (6 mm) were collected at 0.5-hour, 4-hours, and 24-hours from the intradermal *Aspergillus* (4000 PNU/ml) injections from each horse. For the 0.5 and 4-hour skin biopsies, the samples were taken from the intradermal injections outside the test grid. For the 24- hour skin biopsies, the highest concentration of *Aspergillus* was identified within the test grid (after the 24 hour measurement had been recorded) and

subsequently taken from within the test grid. This was done to reduce the number of total intradermal injections. Lidocaine 2% (1 ml) was injected subcutaneously and a 6 mm skin punch biopsy device was used to collect the samples. The biopsy site was closed with a simple interrupted suture. The samples were preserved in 70% ethanol for a minimum of 24 hours. The samples where then cut in half, routinely fixed in paraffin blocks, cut at a thickness of 5 µm with a rotary microtome, and stained with hemotoxylin and eosin (H&E). The biopsy sections were subjectively evaluated microscopically for the presence of inflammation, predominate cellular infiltrate, and presence of edema. A subjective scale was followed by a pathologist who examined all slides. The grading system for inflammation and edema was: 0-none, 1-mild, 2-modest, 3-moderate, and 4-severe.

[a] **Histamine,** Sigma-Aldrich, St. Louis, MO
[b] **Compound 48/80**, Greer Labs, Lenoir, NC
[c] **Phaseolus vulgaris,** Sigma-Aldrich, St. Louis, MO
[d] ***Aspergillus* Mix**, Greer Labs, Lenoir, NC
[e] **Xylazine**, Fermenta Animal Health Co., Kansas City, MO

Table 1 – Intradermal Skin Testing Stimulants and Concentrations

Histamine (mg/ml)	0.02	0.004	0.0008	0.00016	0
48/80 (µg/ml)	50	25	-----	-----	0
PHA (mg/ml)	0.5	0.2	0.08	0.032	0
Aspergillus (PNU/ml)	4000	2000	1000	500	0

* 1 intradermal syringe of each concentration of each stimulant on each side of the neck = 18 injections/side of the neck**.** (Protein Nitrogen Units = PNU)

Statistical Anaylsis for Compound 48/80 and Aspergillus

Upon inspection of the raw data, measurable wheal responses for compound 48/80 were at the 0.5-hour time while *Aspergillus* was evaluated at the 0.5-, 4- and 24-hour times. Concentration response curves were made for the two stimulants at the appropriate times (0.5hrs, 4hrs and/or 24hrs). Simple linear models were used to model the relationship between concentration of stimulant/antigen and response (wheal diameter) for compound 48/80 and *Aspergillus*.

Parameters estimated using the simple linear models were subject to ANOVA. The ANOVA was used to test for effects of RAO on the slope. Specifically, the slope of the line [b] of normal and RAO affected horses was evaluated for differences in rate of rise to detect if normal and RAO affected horses responded differently.

Results for Compound 48/80 and Aspergillus

Daily physical exams were within normal limits. All horses tolerated intradermal skin testing with only minor discomfort observed during the procedure. No significant or severe reactions were noted to any of the injections. All control injections produced 9-11 mm wheals that were gone by the 4-hour reading.

A simple linear model was used to represent compound 48/80. No statistical differences were noted between normal and RAO affected horses when the compound 48/80 data was subject to ANOVA at the 0.5-hour time. Because compound 48/80 did not demonstrate any differences between the normal and RAO affected horses and did not have a concentration affect, no further figures or evaluations were made. *Aspergillus* was also represented by a simple linear model (Figures 1-3). *Aspergillus* data was subject to ANOVA at the 0.5- and 4-hour times with no differences detected between

normal and RAO affected horses. However, none of the normal horses had wheals at the 24-hour time whereas 4 of 5 RAO affected horses had measurable wheals at the 24-hour time at the highest concentration of *Apsergillus* (Table 2). *Aspergillus* therefore had a concentration affect on RAO affected horses (P value = 0.0015) that was not observed in normal horses.

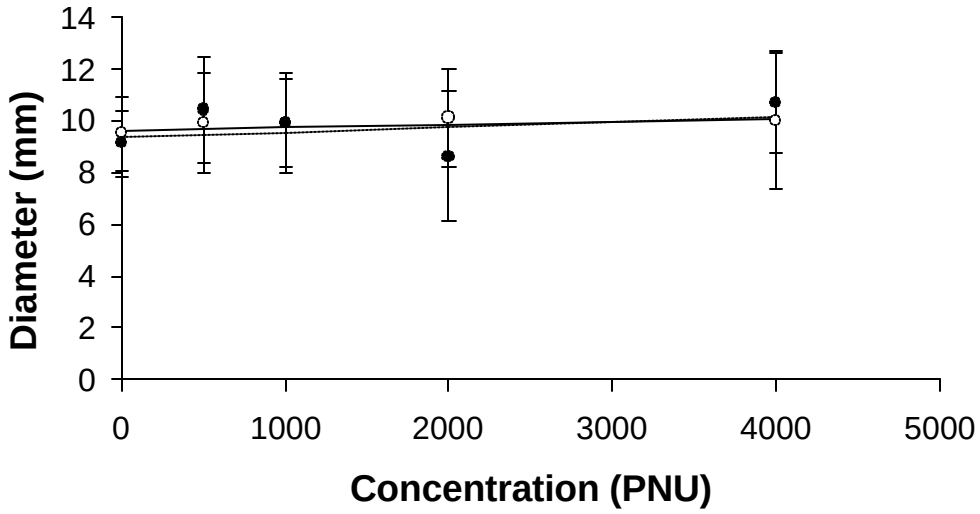


Figure 1: Model of Aspergillus (0.5-hour). Linear models of the mean wheal diameters of 10 horses (total = 20 wheals/concentration) 0.5 hours after intradermal injection of 5 concentrations of *Aspergillus.* Open circles represent mean wheal diameters for normal horses; Filled circles represent mean wheal diameters for RAO horses. Solid line represents the fitted model for *Aspergillus* for normal horses; Dashed line represents fitted model for RAO affected horses.

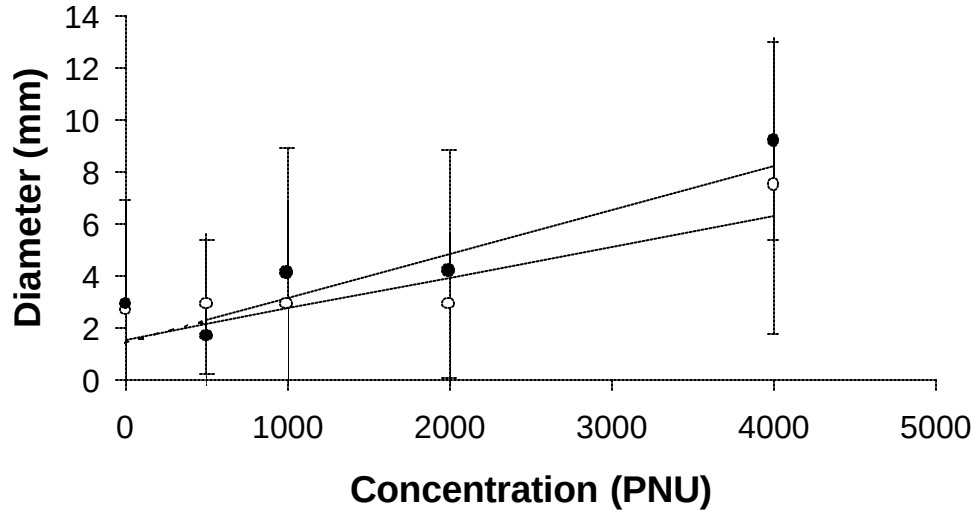


Figure 2: Model of Aspergillus (4-hours). Linear models of the mean wheal diameters of 10 horses (total = 20 wheals/concentration) 4 hours after intradermal injection of 5 concentrations of *Aspergillus.* Open circles represent mean wheal diameters for normal horses; Filled circles represent mean wheal diameters for RAO horses. Solid line represents the fitted model for *Aspergillus* for normal horses; Dashed line represents fitted model for RAO affected horses.

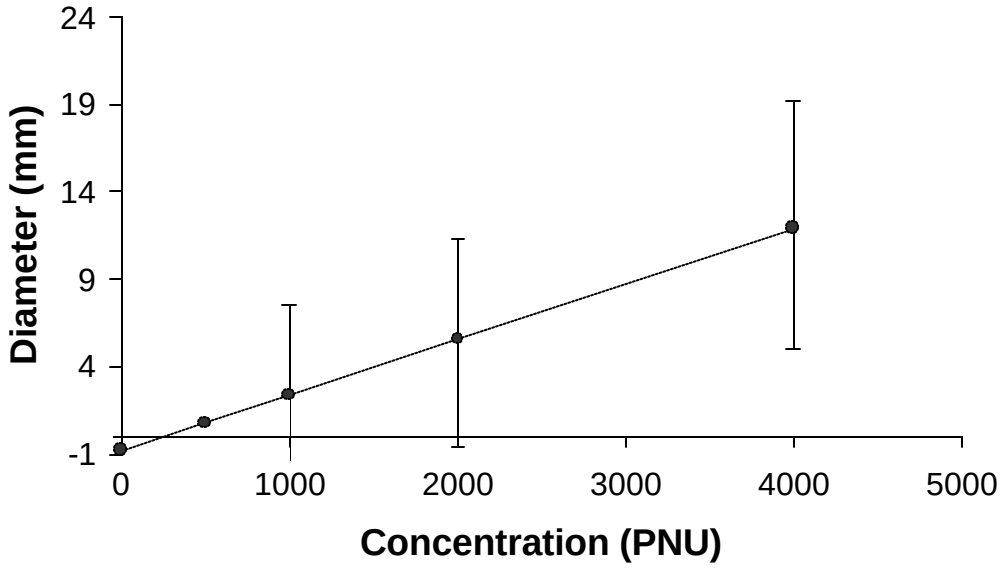


Figure 3: Model of Aspergillus (24-hours). Linear model of the mean wheal diameters of 5 ROA affected horses (total = 10 wheals/concentration) 24 hours after intradermal injection of 5 concentrations of *Aspergillus*. Filled circles represent mean wheal diameters for RAO horses. Dashed line represents the fitted model for *Aspergillus* for RAO affected horses. No wheal responses noted in normal horses.

Table 2: **Analysis of Intercept and Slope of Normal and RAO affected horse for *Aspergillus*.** The intercept (a) represents the mean of all wheals (total 10 wheals) created by the intradermal injection of Aspergillus at the 0 (control) concentration at the indicated times in 5 normal horses and 5 ROA affected horses. The slope represents the rate at which the wheal size increases across increasing concentrations of *Aspergillus*. STD-standard deviation.

	0.5 hours		4 hours		24 hours	
	Intercept (a)	Slope (b)	Intercept (a)	Slope (b)	Intercept (a)	Slope (b)
Control	9.634	0.00009	1.568	0.001	NA	NA
RAO	9.329	0.0002	1.428	0.002	-0.783	0.00318
P-Value	0.616	0.657	0.926	0.274	NA	NA

NA-Not Applicable; No response at the 24 hour time for normal horses.

Histologic Examination of Aspergillus

Based on examination of the raw data and the presence of wheal reactions 24-hours after intradermal injection of *Aspergillus* (4000 PNU/ml) in RAO affected horses, skin biopsy samples of the normal and RAO-affected horses were examined microscopically at this time (Table 3). All samples from RAO affected horses demonstrated mild to modest edema of the deep dermis; all samples from the normal horses demonstrated no edema. Two of the 4 ROA affected horses (horses 2 & 6) demonstrated moderate perivascular lymphohistiocytic inflammation along with perivascular eosinophils and few neutrophils (Figures 6-10). In contrast, the other 2 of the 4 ROA affected horses (horses 4 & 8) demonstrated mild perivascular exudation of neutrophils with few mononuclear cells and eosinophils noted. Three of the 4 normal horses (horses 3,7, & 10) demonstrated minimal inflammation with occasional perivascular neutrophils noted. One of the 4 normal horses (horse 1) demonstrated a moderate neutrophilic perivascular inflammation (Figures 4-5).

Table 3: Histologic evaluation of biopsy sites 24 hours after intradermal injection of *Aspergillus* grading edema and inflammation for normal and RAO affected horses. Grading scale: 0-none, 1-mild, 2-modest, 3-moderate, 4-severe, NA-not applicable. The character of inflammation is noted were applicable; MO-macrophage, LO-lymphocyte, NO-neutrophil, EO-eosinophil.

Horse	Stimulant	Time (hrs)	RAO	Edema	Inflammation	Character of Inflammation
1	*Aspergillus*	24	No	0	3	Neutrophilic
3	*Aspergillus*	24	No	0	0	No Cells
7	*Aspergillus*	24	No	0	0	No Cells
10	*Aspergillus*	24	No	0	0	No Cells
2	*Aspergillus*	24	Yes	1	3	Mixed; MO, LO, NO, EO
4	*Aspergillus*	24	Yes	1	1	Mixed; NO, MO, EO
6	*Aspergillus*	24	Yes	2	3	Mixed; MO, LO, EO, NO
8	*Aspergillus*	24	Yes	1	2	Mixed; NO, MO, LO, EO

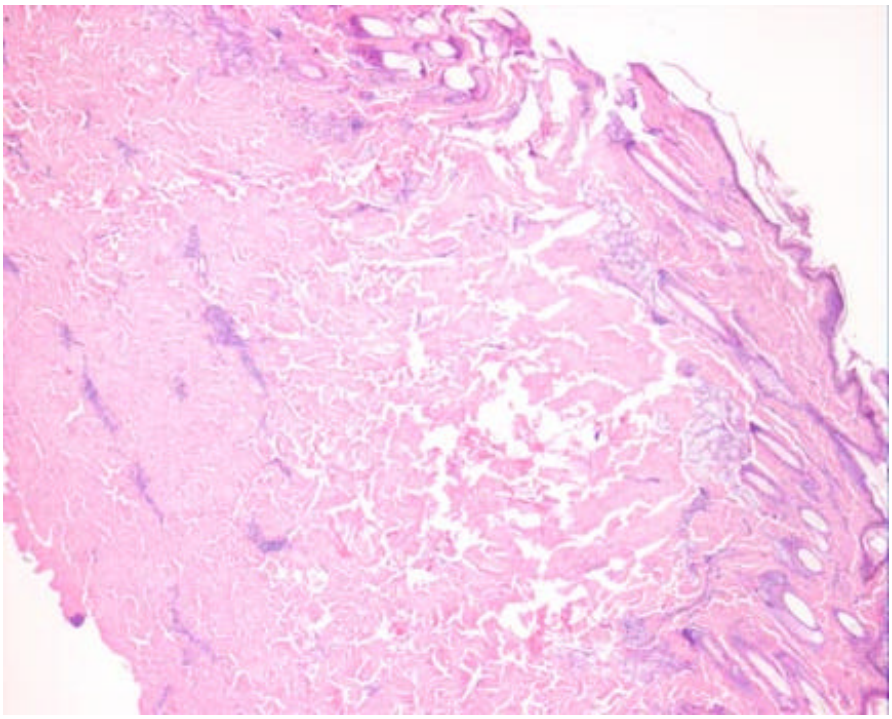


Figure 4: Photomicrograph of a biopsy site from a normal horse (horse 1) 24 hours after intradermal injection of *Aspergillus* (4000 PNU/ml) demonstrating moderate perivascular inflammation. H&E Stain; Magnification – 4x.

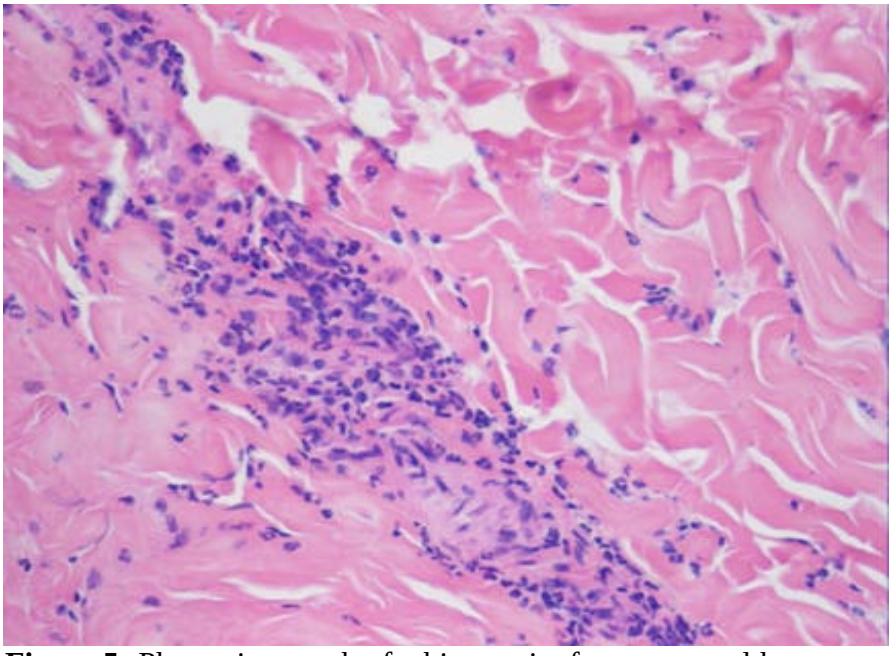


Figure 5: Photomicrograph of a biopsy site from a normal horse (horse 1) 24 hours after intradermal injection of *Aspergilllus* (4000 PNU/ml) demonstrating moderate perivascular neutrophilic inflammation. H&E Stain; Magnification – 40x.

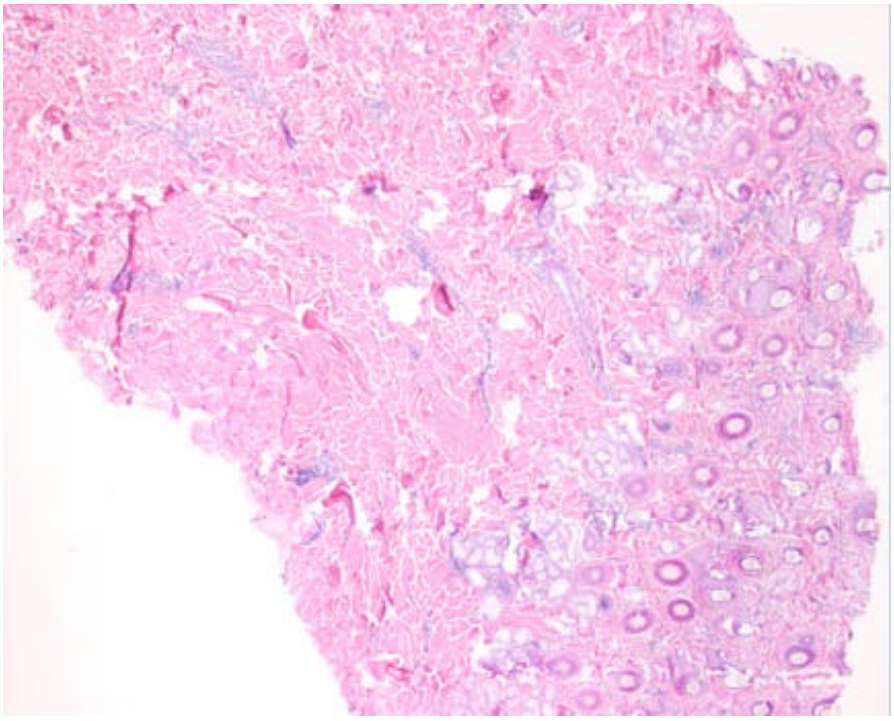


Figure 6: Photomicrograph of a biopsy site from a RAO affected horse 24 hours after intradermal injection of *Aspergillus* (4000 PNU/ml) demonstrating moderate perivascular inflammation. H&E Stain; Magnification – 4x.

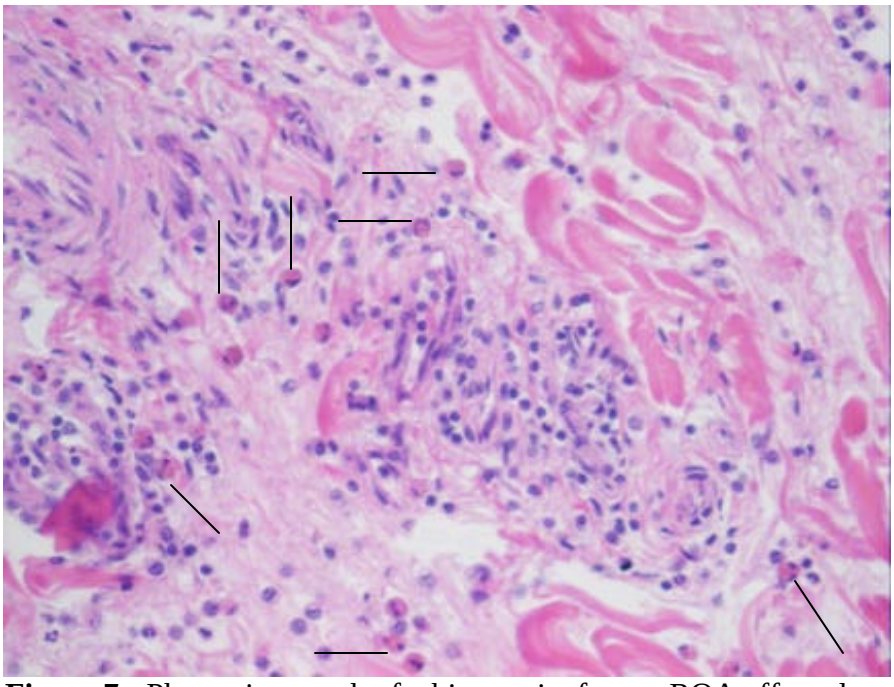


Figure 7: Photomicrograph of a biopsy site from a ROA affected horse 24 hours after intradermal injection of *Aspergillus* (4000 PNU/ml) demonstrating a mixed, lympho-histiocytic, perivascular inflammation along with eosinophils (arrows) and neutrophils. H&E Stain; Magnification – 40x.

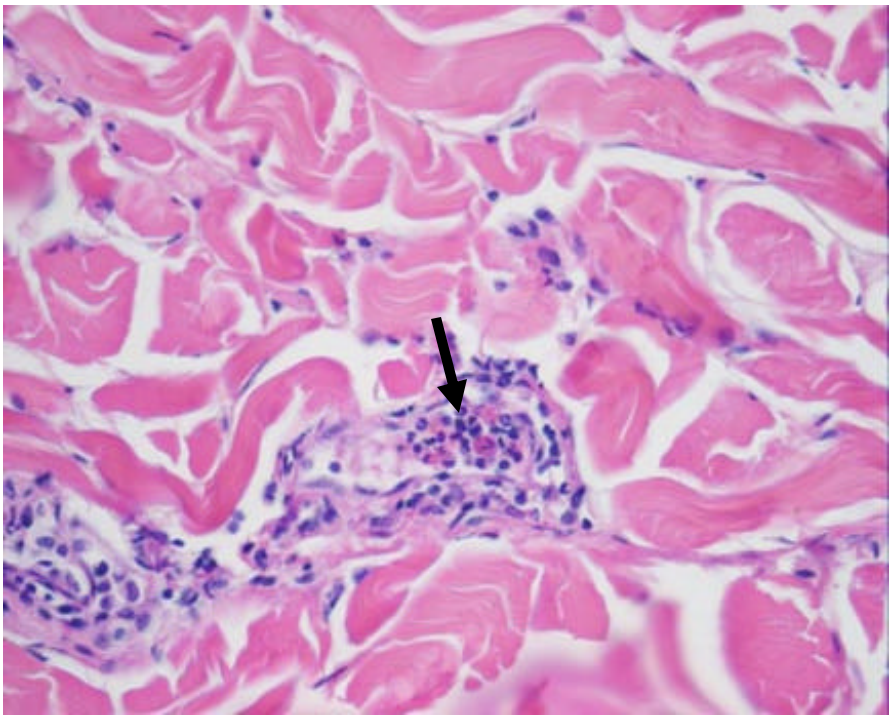


Figure 8: Photomicrograph of a biopsy sample from a ROA-affected horse 24 hours after intradermal injection of *Aspergillus* (4000 PNU/ml) demonstrating margination of eosinophils (arrow) and mononuclear cells. H&E Stain; Magnification – 40x.

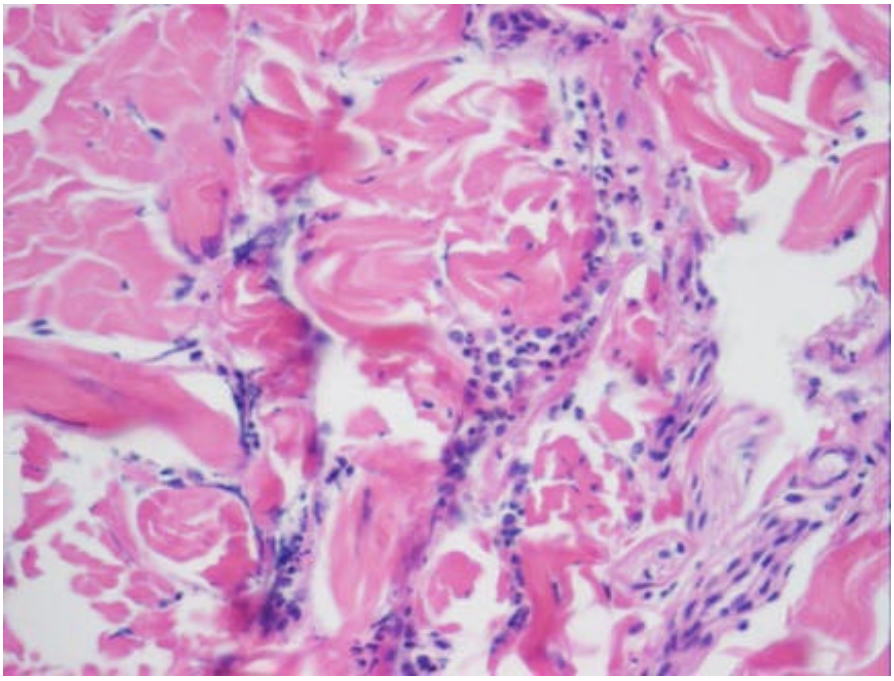


Figure 9: Photomicrograph of a biopsy sample from a RAO-affected horse 24 hours after intradermal injection of *Aspergillus* (4000 PNU/ml) demonstrating exudation of neutrophils and monocytes. H&E Stain; Magnification – 40x.

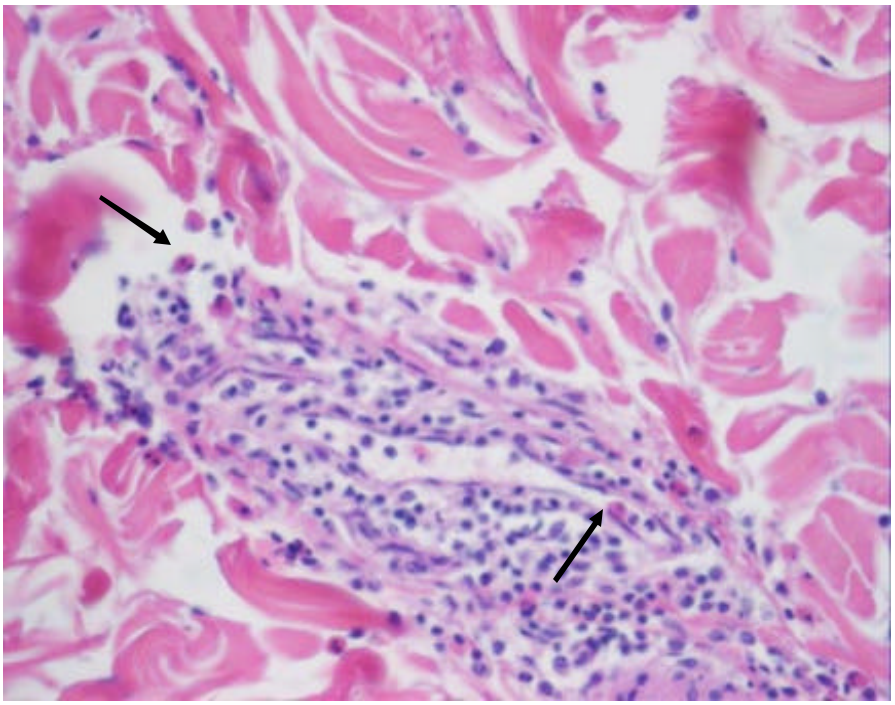


Figure 10: Photomicrograph of a biopsy site from a ROA-affected horse 24 hours after intradermal injection of *Aspergillus* (4000PNU/ml) demonstrating margination and exudation of mononuclear cells, neutrophils and eosinophils (arrows). H&E Stain; Magnification – 40x.

Statistical Analysis of Histamine and PHA

The relationships between stimulant concentrations and wheal diameters were modeled for histamine and PHA to evaluate the rate of rise [b] and the maximum wheal diameter (plateau [a]) for the normal and RAO affected horses. The rate of rise [b] of the line was interpreted as the degree of sensitivity (i.e. hyper/hyposensitive) while the plateau [a] was interpreted as responsiveness (i.e. hyper/hyporesponsive) (Figure 11). Upon inspection of the raw data, histamine was evaluated at the 0.5- and 4-hour times while PHA was evaluated at the 4-and 24-hour times.

The data for histamine and PHA did not fit a simple linear model; therefore, the Chapman-Richard's equation, $Y = a[1-e^{-bX}]^c$, was used to model the relationship between concentration of stimulant and response (wheal diameter). Parameters estimated using the concentration-response models were subjected to ANOVA. The ANOVA was used to test for effects of RAO on sensitivity and responsivity. Specifically, the plateau [a] of normal and ROA affected horses was evaluated for differences in wheal diameter to detect if ROA affected horses were hyporesponisive or hyperresponsive in relationship to normal horses. The rate of rise [b] of normal and ROA affected horses was evaluated for differences in rate of rise to detect if ROA affected horses were hyposensitive or hypersensitive in relationship to normal horses (Figure 11).

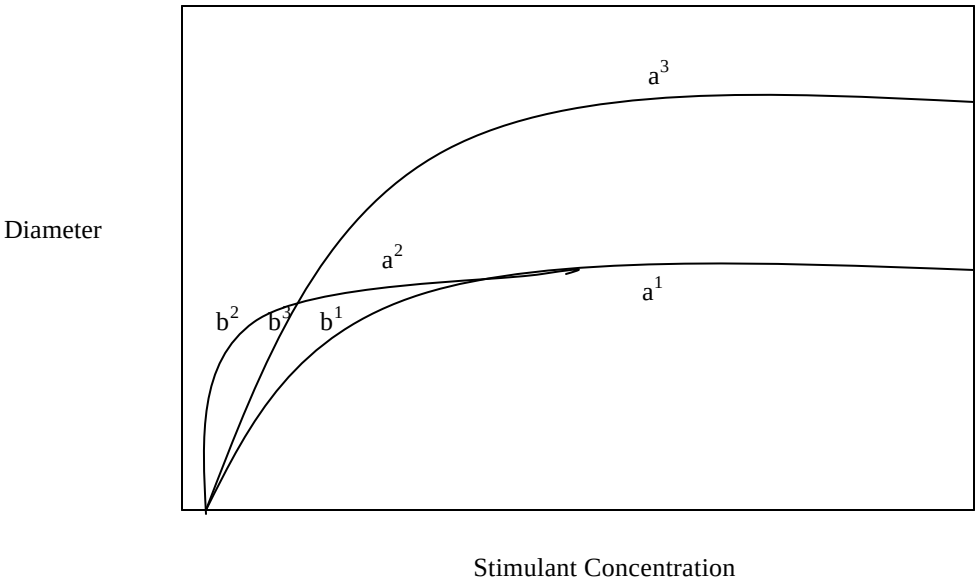


Figure 11: Diagrammatic representation of the Chapman Richard's Model used for histamine and PHA. The wheal response to intradermal injection of antigen is demonstrated in a normal horse (a^1=plateau, b^1=rate of rise), a hypersensitive horse (a^2, b^2) and a hyper-responsive horse (a^3, b^3). The hypersensitive horse is represented as a steeper rate of rise ($b^2 > b^1$ and b^3) but the same maximum diameter as the normal horse ($a^2=a^1$). The hyper-responsive horse is represented as a greater maximum diameter ($a^3 > a^2$ and a^1) but the same rate of rise as the normal line ($b^1=b^3$).

Results for Histamine and PHA

All horses tolerated intradermal skin testing with histamine and PHA with only minor discomfort observed during the procedure. No severe reactions were noted to the injections; however, the wheals created by PHA were painful on digital palpation at the 4- and 24- hour times. The non-linear (Chapman-Richard's Equation) model was used to represent histamine (Figures 12 and 13) and PHA (Figures 14 and 15). Because of the wide range of histamine concentrations, the models of histamine were placed on a logarithmic scale to provide better representation. The rate of rise [b] and plateau [a] between normal and RAO affected horses was evaluated by ANOVA. A statistical difference was noted in the rate of rise for histamine at the 0.5-hour time between normal and ROA affected horses with the RAO affected horses demonstrating a greater rate of rise (b) when compared to the normal horses. This difference between normal and ROA affected horses was not noted at the 4-hour time for histamine. Two concentrations of PHA were incorrectly labeled in horse 7 and provided erroneous results; therefore, horse 7 was removed from the PHA evaluation at 4 and 24-hours. No statistical differences were detected for PHA between normal and ROA affected horses at the 4- and 24-hour times (Table 4).

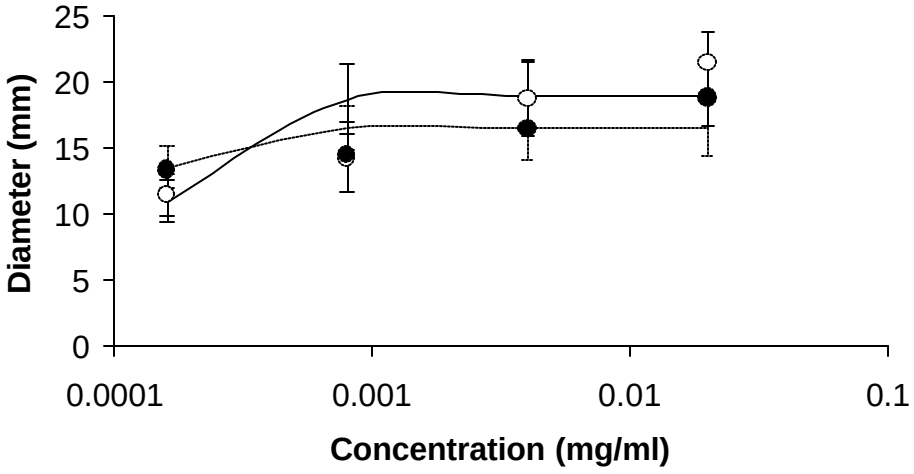


Figure 12: Model of Histamine; Normal and ROA affected horses (0.5-hour). Linear models of the mean wheal diameters of 10 horses (total = 20 wheals/concentration) 0.5 hours after intradermal injection of 5 concentrations of histamine. Open circles represent mean wheal diameters for normal horses; Filled circles represent mean wheal diameters for RAO horses. Solid line represents the fitted model for histamine for normal horses; Dashed line represents RAO affected horses. Model is on a logarithmic scale (note- wheals created by 0 concentration not plotted because of logarithmic scale).

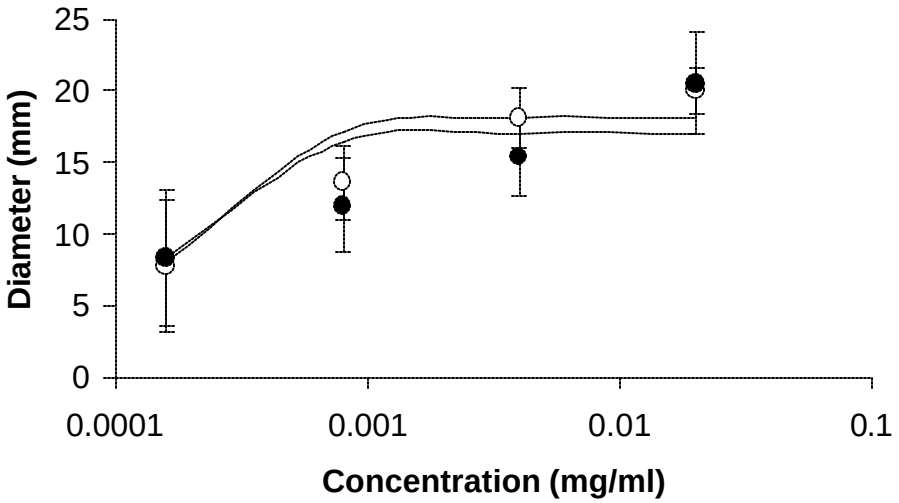


Figure 13: Model of Histamine; Normal and RAO affected horses (4 hour). Linear models of the mean wheal diameters of 10 horses (total = 20 wheals/concentration) 4 hours after intradermal injection of 5 concentrations of histamine. Open circles represent mean wheal diameters for normal horses; Filled circles represent mean wheal diameters for ROA affected horses. Solid line represents the fitted model for histamine for normal horses; Dashed line represents RAO horses. Model is on a logarithmic scale (note-0 concentration not plotted because of logarithmic scale).

Table 4: Analysis of Intercept and Rate of Rise of Normal and ROA affected horses for Histamine. The plateau (a) represents the mean of the maximum wheal diameters created by the intradermal injection of histamine across 5 concentrations of histamine at the 0.5- and 4-hour times in 5 normal horses and 5 RAO affected horses. The rate or rise (b) represents the rate at which the wheal size increases across increasing concentrations of histamine. STD-standard deviation.

	0.5 hours		4 hours	
	Plateau (a)	Rate of Rise(b)	Plateau (a)	Rate of Rise (b)
Normal	18.988	5399.05	18.113	3633.15
RAO	16.536	10800	17.041	4224.69
P-Value	0.067	0.0228	0.419	0.726

NA-Not Applicable; Response too sporadic to be interpreted at this time.

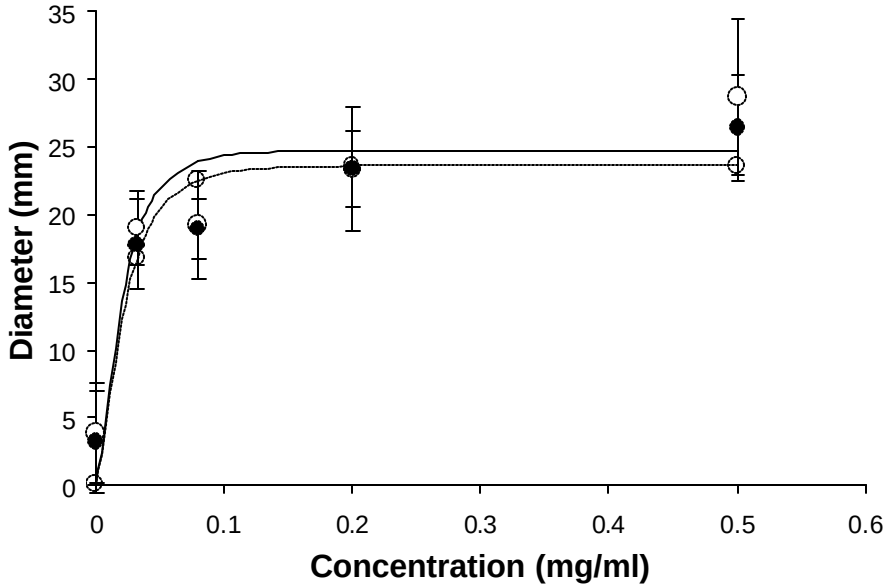


Figure 14: Model of Phaseolus vulgaris; Normal and ROA affected horses (PHA-4 hours). Non-linear model of the mean wheal diameters of 9 horses (total = 18 wheals/ concentration) 4 hours after intradermal injection of 5 concentrations of PHA. Open circles represent mean wheal diameters of normal horses; Filled circles represent mean wheal diameters of ROA affected horses. Solid line represents the fitted model for normal horses; Dashed line represents model for RAO affected horses.

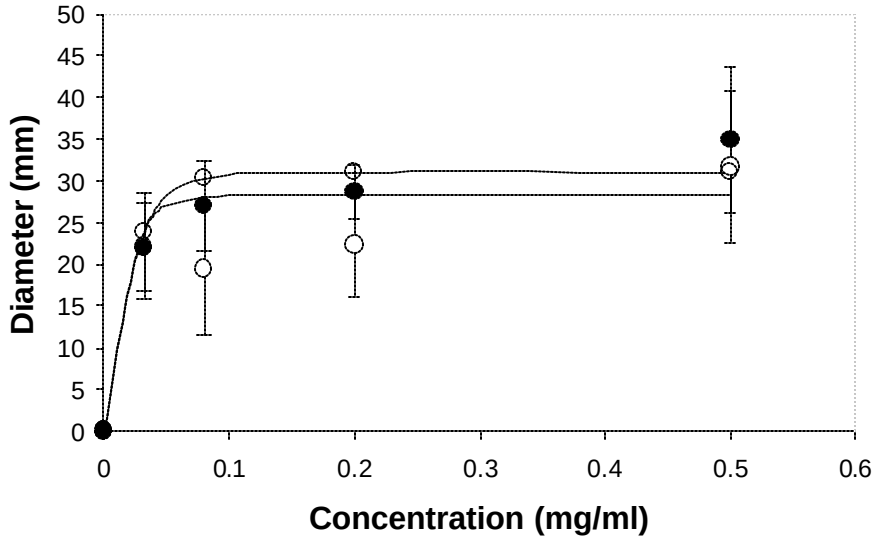


Figure 15: Model of Phaseolus vulgaris; Normal and RAO affected horses (PHA-24 hours). Non-linear model of the mean wheal diameters of 9 horses (total = 18 wheals/ concentration) 24 hours after intradermal injection of 5 concentrations of PHA. Open circles represent mean wheal diameters for normal horses; Filled circles represent mean wheal diameters for RAO affected horses. Solid line represents the fitted model for normal horses; Dashed line represents the model for RAO affected horses.

Table 5: Analysis of Plateau and Rate of Rise of Normal and RAO affected horse for Phaseolus vulgaris. The plateau (a) represents the mean of the maximum wheal diameters created by the intradermal injection of PHA across 5 concentrations of PHA at the 4- and 24-hour times in 4 normal horses and 5 RAO affected horses. The rate or rise (b) represents the rate at which the wheal size increases across increasing concentrations of PHA. STD-standard deviation.

	4 hours		24 hours	
	Plateau (a)	Rate of Rise (b)	Plateau (a)	Rate of Rise (b)
Normal	24.699	42.882	28.868	59.026
RAO	25.602	38.550	31.094	45.371
P-Value	0.646	0.707	0.485	0.610

Discussion

Intradermal skin testing has been used extensively in human medicine to identify causative antigens in various allergic disorders such as asthma, insect hypersensitivity and allergic rhinitis. The IDST has been extrapolated to equine medicine in an attempt to identify causative antigens in ROA affected horses. To date, many of the published studies in regards to equine intradermal skin testing and RAO have been conflicting.

Aspergillus has been implicated as a causative antigen in the development of RAO in the horse [40]. In this study, no statistically significant differences in intercept [a] or slope [b] between normal horses and RAO affected horses were found when evaluating *Aspergillus* at the 0.5- and 4-hour time. However, 4 of 5 RAO affected horses did demonstrate wheals (range 12-22 mm) at the 24-hour time at the highest concentration (4000 PNU/ml). No wheal reactions were noted in the normal horses at the corresponding time and concentration. Interestingly, the RAO group did not demonstrate any difference from the normal group at the 0.5- or 4-hour time when intradermal skin test results are commonly evaluated. This information supports a prior study by Evans *et al*. in which approximately 5.5% of positive skin test reactions were noted at 24 hours in the ROA affected horses [85]; therefore, the information from this study suggests that skin testing should be assessed at 24-hours as well as 0.5- and 4-hours. This information also suggests that the intradermal reaction to *Aspergillus* may be of a delayed type or late-phase reaction. A delayed type IV reaction was supported in 2 of the 4 RAO affected horses by the presence of a monouclear inflammatory response similar to PHA with the additional presence of eosinophils. No inflammatory response was noted in 3 of 4 normal horses 24 hours after intradermal injection of Aspergillus (4000 PNU/ml)

whereas one of the normal horses demonstrated a moderate neutrophilic inflammatory response.

In a study by McPherson *et al*., the investigators found that skin reaction to intradermally injected *Aspergillus* in RAO affected horses results in a dual hypersensitivity response; a weak response shortly after injection followed by an Arthus-like response 4-8 hours later [40]. In contrast, Lorch *et al*. performed IDST on 22 normal horses and 16 RAO affected horses [84]. The investigators stated that positive reactions were uncommon at the 24-hour time accounting for 2% of positive reactions in normal horses (14/709) and 4% of positive reactions in RAO affected horses (13/370). Of the positive reactions at the 24-hour time, the normal horses reacted exclusively to mold allergens (*Candida albicans, Rhizopus mix, Pullularia pullulans*) while the RAO affected horses reacted positively to mold, grain mill dust mix, dock-sorrel mix, and black ant extract [84].

Interestingly, in this study, variable numbers of eosinophils among a mixed cellular pattern were a consistent histologic finding in the skin biopsy sites of RAO affected horses intradermally injected with 4000 PNU/ml of *Aspergillus* at 24 hours. Eosinophils are attracted to areas of mast cell degranulation and are a characteristic component of type I hypersensitivity reactions [1]. Eosinophils have also been noted among mixed cellular responses in IDST during the late phase reaction in human studies [29, 66]. The presence of eosinophils and other inflammatory cells in the dermis have been demonstrated in biopsy samples from horses with *Culicoides* hypersensitivity reactions [21]. To the author's knowledge, this is the first study that has examined skin

biopsy samples in RAO affected horses after intradermal injection of the causative antigen, *Aspergillus* mix.

Previous intradermal skin test studies in horses have used a maximum dose of 0.1 to 0.05 mls of *Aspergillus* at a concentration of 1000 PNU/ml [82, 84, 102]. This study suggests that higher doses must be used in IDST to achieve a wheal response in RAO affected horses with this antigen. Although one group of investigators have stated that excessively high concentrations of antigen may elicit false positive dermal responses, this study suggests that an *Aspergillus* concentration of 4000 PNU/ml is not excessive based on the fact that no wheals were noted in the control group [89].

RAO was confirmed in the RAO group by exposing the affected horses to moldy hay prior to the initiation of this study. Clinical signs of RAO were noted within 3 days of exposure to moldy hay as evidenced by tachypnea, increased respiratory effort and wheezes/crackles upon auscultation of the lung fields. A sample of the moldy challenge hay was submitted to a diagnostic laboratory (Greer Laboratories, NC) and evaluated for content. Within the samples of hay, both *Aspergillus* and *Micropolyspora* species were identified implicating them as environmental antigens leading to RAO in the horses used in this study. Previous reports have suggested that *Aspergillus* and *Micropolyspora* are involved in the pathogenesis of RAO based on aerosol challenge and hay exposure [39, 40]. The exact reason why 1 of the 5 RAO-affected horses in this study did not demonstrate positive intradermal skin test reactions to *Aspergillus* is not known. *Micropolyspora* may have been the causative antigen that caused clinical RAO in this horse during hay challenge. It is also possible that the IDST may not be an indicator of causative antigens (i.e. *Aspergillus*) in all RAO affected horses. This is supported by the

findings of McPherson *et al.* in that disagreement was found between positive IDST results with *Aspergillus fumigatus* and inhalation challenge in RAO affected horses [40]. Another group of investigators suggest that the lack of correlation between bronchial and dermal reactivity may be attributed to local production of allergen specific IgE within the respiratory tract or skin and/or regional heterogeneity in mast cell responsivness [89].

In this study, a statistical difference in the slope [b] of histamine between normal and RAO affected horses was identified at the 0.5-hour time. The rate of rise to intradermal injection of histamine was more rapid in the RAO affected horses suggesting the RAO affected horses are hypersensitive in comparison to normal horses. Dirscherl *at al*. demonstrated that the sensitivity of basophils was increased in RAO affected horses while McGorum *et al*. demonstrated increased numbers of mast cells/basophils in BAL fluid and increased concentrations of histamine in pulmonary epithelial lining fluid in RAO affected horses at 5 hours post-hay challenge [36, 39]. These studies suggests that RAO affected horses have increased mast cell/ basophil sensitivity to specific antigens and undergo a late-phase hypersensitivity reaction in which increased amounts of histamine are liberated from mast cells/basophils after antigen challenge.

PHA did not result in any significant differences in slope [b] or plateau [a] between normal and RAO affected horses. This information supports another study in which no differences were found to *in vitro* stimulation of lymphocytes by PHA between normal and RAO affected horses [36]. This information suggests that PHA may be an appropriate control stimulant in equine intradermal skin testing. No statistically significant differences in intercept [a] or slope [b] between normal and RAO affected horses was found when evaluating compound 48/80. However, the usefulness of this

information is questionable as an inappropriately low concentration of compound 48/80 may have been used (as discussed in chapter 2).

Conclusions

We conclude that *Aspergillus* species may be found in poorly cured hay and the presence of *Aspergillus* in hay fed to RAO affected horses may induce clinical signs of RAO. In addition, *Aspergillus* found in hay may correlate with positive intradermal skin test wheals in RAO affected horses possibly implicating *Aspergillus* as a causative antigen in some horses afflicted with RAO. Horses with RAO also had a steeper slope to histamine in comparison to normal horses which suggests that RAO affected horses are hypersensitive to intradermal injection of histamine. No significant differences in concentration response were detected in the positive control stimulants compound 48/80 and PHA.

SUMMARY:

The original objectives of the first part (Chapter 2) of this study were to: 1) evaluate the variance in wheal diameters created by repeated intradermal injections of the three stimulants at 5 concentrations within a horse, 2) evaluate the variance in wheal diameters created by repeated intradermal injections of the three stimulants at 5 concentrations between 12 horses, 3) establish at what time points the wheal responses to each stimulant are best evaluated, and 4) histologically evaluate skin biopsy samples from the wheals created by intradermal injection of each stimulant at three different times.

The first part of this study demonstrated that there is minimal variance in the wheals produced by intradermal injection of positive control stimulants, histamine, compound 48/80, and PHA, within a normal horse and between normal horses. Minimal variance was noted to intradermal injection of histamine at the 0.5-hour time. This study also suggested that histamine is best evaluated at the 0.5- and/or 4-hour time depending on the concentration of histamine utilized. Minimal variance was noted to intradermal injection of compound 48/80 at the 0.5-hour time; compound 48/80 was best evaluated at the 0.5-hour time. However, a concentration response curve was not noted at the concentrations of compound 48/80 used in this study. Therefore, a higher concentration of compound 48/80 than the maximum dose used in this study (50 µg/ml) may be necessary to cause mast cell degranulation. PHA demonstrated minimal variance to intradermal injection at the 4-hour time but a substantial amount of wheal variance was noted at 24-hours. PHA was best evaluated at 4- and 24-hours. In addition, this study found that intradermal injection of histamine caused severe dermal edema and

margination of neutrophils and eosinophils at 0.5 hours. Compound 48/80 demonstrated mild to modest dermal edema at 0.5 hours while PHA demonstrated severe dermal edema, hemorrhage, and lymphactic ectasia at 4 and 24 hours. PHA also demonstrated a neutophilic inflammation at 4 hours that progressed to a mixed lymphohistiocytic and neutophilic inflammation at 24 hours.

The original objectives of this second part (Chapter 3) of this study was to: 1) compare wheal reactions between normal horses and horses with RAO using the three known positive control stimulants, histamine, compound 48/80, and Phaseolus vulgaris (PHA), along with the environmental antigen *Aspergillus* and 2) compare, histologically, the cellular response created by the intradermal injection of *Aspergillus* between normal horses and horses with RAO.

This second part of study demonstrated a notable response to the intradermal injection of *Aspergillus* in RAO affected horses at 24-hours at a concentration of 4000 PNU/ml when compared to normal horses. This suggests that the IDST may be an effective diagnostic test in the identification of causative antigens in RAO affected horses. However, the IDST was non-diagnostic for 1 of 5 RAO affected horses and therefore, may not be completely reliable. Further studies evaluating additional antigens and larger numbers of RAO affected horses are necessary to determine the sensitivity and specificity of the IDST in identifying causative antigens. This study also demonstrated no edema and modest to moderate neutophilic inflammation in normal horses after intradermal injection of *Aspergillus* at 24 hours. In contrast, RAO affected horses demonstrated mild to modest edema and a mild to moderate mixed inflammatory

response (lympho-histocytic, neutrophilic, eosinophilic) after intradermal injection of Aspergillus at 24 hours.

Collectively, this information suggests that the intradermal skin test is a repeatable diagnostic test in horses and that there may be a relationship between the presence of *Aspergillus* in hay, positive intradermal skin test results using *Aspergillus*, and the induction of clinical RAO in RAO affected horses.

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Appendix A: Histamine - Mean wheal diameters. Mean wheal diameters produced by 4 intradermal injections of histamine at 5 concentrations (0, 0.00004, 0.0002, 0.001, 0.005 mg/ml) at 0.5, 4, and 24 hours in 12 horses. Experiment 1; Normal horses.

	0.5 hour					4 hour					24 hour				
	0	0.00004	0.0002	0.001	0.005	0	0.00004	0.0002	0.001	0.005	0	0.00004	0.0002	0.001	0.005
			(mg/ml)					(mg/ml)					(mg/ml)		
Horse 1	10.25	10	11	14	15.75	0	0	3.25	10.75	16.5	0	0	0	0	0
Horse 2	10	9.75	10.75	11.75	14.5	0	2.5	6	8.5	15.75	0	0	0	0	0
Horse 3	9.25	9.25	10	11.75	14.75	0	0	5.75	8.25	13	0	0	0	0	0
Horse 4	9.33	9.5	9	12.75	14.75	0	0	2.25	10.75	12.75	0	0	0	0	0
Horse 5	9.75	10	10	11.25	15.5	0	0	2.5	7.5	11.75	0	0	0	0	0
Horse 6	9.5	9.25	10.5	11.5	15	0	0	0	6	11.5	0	0	0	0	0
Horse 7	10.25	9.5	10.25	11.75	15.25	0	0	0	8.5	13.25	0	0	0	0	0
Horse 8	9.5	10	10.5	13.25	16.75	0	0	0	6.5	12.75	0	0	0	0	0
Horse 9	10.25	10.25	10.5	14.5	18.25	0	0	0	2.75	15.75	0	0	0	0	0
Horse 10	10.75	10	9.5	11.25	14.5	0	0	0	0	12.25	0	0	0	0	0
Horse 11	9.5	9	9.75	12.5	14	0	2.5	2.5	8.25	11.25	0	0	0	0	0
Horse 12	9.75	9.75	12	14.25	19	0	0	2.5	9.5	10.25	0	0	0	0	0

Appendix B: Compound 48/80 - Mean wheal diameters. Mean wheal diameters produced by 4 intradermal injections of compound 48/80 at 5 concentrations (0, 12.5, 25, 37.5, 50 µg/ml) at 0.5, 4, and 24 hours in 12 horses. Experiment 1; Normal horses.

	0.5 hour					4 hour					24 hour				
	0	12.5	25 (µg/ml)	37.5	50	0	12.5	25 (µg/ml)	37.5	50	0	12.5	25 (µg/ml)	37.5	50
Horse 1	9.75	9.25	10	10.25	10	0	0	0	0	0	0	0	0	0	0
Horse 2	7.25	9.5	9.5	10	7.25	5	0	0	0	0	0	0	0	0	0
Horse 3	9.25	9.25	10.25	9.5	9.25	0	0	0	0	0	0	0	0	0	0
Horse 4	9.5	9.3	9.5	9.5	10	2.25	2.5	0	0	4.75	0	0	0	0	0
Horse 5	9.75	9.75	10.25	9.75	10.75	2.75	2.5	0	0	0	0	0	0	0	0
Horse 6	9.75	10	9.75	9.5	10.25	0	0	0	0	0	0	0	0	0	0
Horse 7	7.5	9	9.75	9.75	9.25	0	0	0	0	0	0	0	0	0	0
Horse 8	9.75	9.25	11	10.5	12	0	0	0	0	50	0	0	0	0	0
Horse 9	9.75	10.25	10.25	11.25	12	0	0	0	0	5.25	0	0	0	0	0
Horse 10	9	9.5	10.75	10	10.75	0	0	3	0	0	0	0	0	0	0
Horse 11	9.5	9.5	9.75	10.25	9.75	0	2.25	2.75	2.5	7.5	0	0	0	0	0
Horse 12	10.75	9.25	11	11.25	10.5	0	0	0	37.5	0	0	0	0	0	0

Appendix C: Phaseolus vulgaris (PHA) - Mean wheal diameters. Mean wheal diameters produced by 4 intradermal injections of PHA at 5 concentrations (0, 0.1, 0.4, 0.7, 1 mg/ml) at 0.5, 4, and 24 hours in 12 horses. Experiment 1; Normal horses.

	0.5 hour					4 hour					24 hour				
	0	0.1	0.4 (mg/ml)	0.7	1	0	0.1	0.4 (mg/ml)	0.7	1	0	0.1	0.4 (mg/ml)	0.7	1
Horse 1	9.75	10	10.75	10.5	11.25	0	18.25	26.25	30	34.75	0	17.75	33.5	34.5	50.5
Horse 2	9.5	9.5	9.75	10.5	10.25	0	19.25	24.5	27.5	33.75	0	23.75	40	37.5	48.25
Horse 3	9.25	9.5	9	8.5	9	0	16.5	24	26.75	28	0	10.5	16.25	23.75	17.75
Horse 4	9	9	9	9.5	11	0	21.5	28.25	29.5	31.5	0	12.75	34.75	40.5	52.5
Horse 5	10.3	9.5	10	10.25	9.5	2.5	23.25	29.5	30.5	35.5	0	30.67	15.67	11	0
Horse 6	7.25	10	7	10	8.3	0	18	26.5	26.75	32.75	0	26.75	35.5	42.75	53
Horse 7	9.75	9.75	10.25	10.25	9.5	0	18.25	29.5	30	31.5	0	16	29.25	32	35.3
Horse 8	9.25	10	10.25	10.75	10.75	0	16.75	24	26.5	27.75	0	13.75	19	25	29.25
Horse 9	10.5	14	16.25	16.75	17.5	0	25	34	38.5	41.25	0	25	40.75	51.25	50.5
Horse 10	9	9.5	10.5	10	9.5	0	15.5	24.75	27.75	32.5	0	9	24.5	19.5	28
Horse 11	9.25	9.25	10.25	9	9.25	0	13.75	21	20.25	22.5	0	19.5	23.5	26.75	35
Horse 12	10	10	9.75	9.75	10.25	0	24.75	32.75	33.5	41.25	0	22	44.75	48.75	56.25

Appendix D: Histamine - Mean wheal diameters. Mean wheal diameters produced by 2 intradermal injections of histamine at 5 concentrations (0, 0.00016, 0.0008, 0.004, 0.02 mg/ml) at 0.5, 4, and 24 hours in 10 horses. Experiment 2; Normal and COPD* affected horses.

	0.5 hour					4 hour					24 hour				
	0	0.00016	0.0008	0.004	0.02	0	0.00016	0.0008	0.004	0.02	0	0.00016	0.0008	0.004	0.02
			(mg/ml)					(mg/ml)					(mg/ml)		
Horse 1	9	12.5	16.5	17.5	20	3	8	15.5	18.5	19.5	0	0	0	0	0
Horse 2*	9.5	11.5	14	17	17.5	3	4	9.5	14.5	17.5	0	0	0	0	0
Horse 3	11	12.5	17	20	20.5	4	3.5	14.5	20	20.5	0	0	0	3	0
Horse 4*	7.5	13	16.5	16	21.5	3	12.5	17.5	18.5	26	0	0	0	0	0
Horse 5	9	10.5	11.5	23	24.5	0	10.5	14	17.5	20	0	0	0	0	0
Horse 6*	12	15	15	17	17.5	5	10	12	14.5	18.5	0	0	0	0	0
Horse 7	12.5	9.5	14	17.5	19.5	0	5.5	13	16.5	18	0	0	0	0	0
Horse 8*	6.5	13.5	14.5	16	18.5	5	10.5	11	14	20.5	0	0	0	0	0
Horse 9*	8	14	12.5	16.5	19.5	0	4.5	10	15.5	20	0	0	0	0	0
Horse 10	7	12.5	12.5	16	23	0	11.5	11	18	22	0	0	0	0	0

Appendix E: Compound 48/80 - Mean wheal diameters. Mean wheal diameters produced by 2 intradermal injections of compound 48/80 at 3 concentrations (0, 25, 50 µg/ml) at 0.5, 4, and 24 hours in 10 horses. Experiment 2; Normal and COPD* affected horses.

	0.5 hour			4 hour			24 hour		
	0	25 (µg/ml)	50	0	25 (µg/ml)	50	0	25 (µg/ml)	50
Horse 1	9.5	10.5	11.5	3.5	5	5	0	0	0
Horse 2*	12	12	12.5	2.5	8.5	8.5	0	0	0
Horse 3	11	11.5	12.5	3.5	0	7	0	0	0
Horse 4*	8.5	10	8	5	10	4.5	0	4.5	0
Horse 5	11	11	12.5	3.5	0	0	0	0	0
Horse 6*	9	10	10	2.5	8.5	9.5	0	0	0
Horse 7	12.5	9.5	8.5	0	0	0	0	0	0
Horse 8*	10.5	10	9	0	0	2.5	0	0	6.5
Horse 9*	10	9.5	10	0	4	10.5	0	0	0
Horse 10	6	9.5	10.5	0	8.5	10.5	0	0	0

Appendix F: Phaseolus vulgaris - Mean wheal diameters. Mean wheal diameters produced by 2 intradermal injections of Phaseolus vulgaris (PHA) at 5 concentrations (0, 0.032, 0.08, 0.2, 0.5 mg/ml) at 0.5, 4, and 24 hours in 10 horses. Experiment 2; Normal and COPD* affected horses.

	0.5 hour					4 hour					24 hour				
	0	0.032	0.08	0.2	0.5	0	0.032	0.08	0.2	0.5	0	0.032	0.08	0.2	0.5
			(mg/ml)					(mg/ml)					(mg/ml)		
Horse 1	10.5	10	12	10.5	8.5	8	22.5	24	23.5	35	0	32.5	32.5	31	47.5
Horse 2*	9.5	9.5	11.5	14.5	15	5.5	21	21	29.5	31	0	26	26	29	30
Horse 3	10.5	11.5	11	12	11	4	18.5	16.5	24	23	0	23	21	20	29
Horse 4*	10.5	12.5	10	12	9.5	8	19	20.5	26	29	0	14	30	30	28.5
Horse 5	10	10	12	10.5	11.5	5	17.5	17.5	24.5	32	0	18	16.5	22	30
Horse 6*	10	10.5	10	10	10	0	20	18.5	22	26	0	26	27.5	31.5	37.5
Horse 7	10	5	10	7.5	11	2.5	17.5	16.5	19	22.5	0	20	10.5	13.5	22
Horse 8*	8	9	9	10	8.5	0	13	16	17.5	21.5	0	22	32.5	27	50
Horse 9*	10	9.5	10	10	14.5	2.5	16	18.5	22	24.5	0	22	19	26	28.5
Horse 10	9	9.5	10.5	8.5	14	0	19	21.5	25.5	31	0	17	17	25	30

Appendix G: Aspergillus - Mean wheal diameters. Mean wheal diameters produced by 2 intradermal injections of *Aspergillus* at 5 concentrations (0, 500, 1000, 2000, 4000 PNU) at 0.5, 4, and 24 hours in 10 horses. Experiment 2; Normal and COPD* affected horses.

	0.5 hour					4 hour					24 hour				
	0	500	1000	2000	4000	0	500	1000	2000	4000	0	500	1000	2000	4000
	(Protein Nitrogen Units/ml)					(Protein Nitrogen Units/ml)					(Protein Nitrogen Units/ml)				
Horse 1	9	11.5	10	10.5	10	6	3.5	6.5	7	10	0	0	0	0	0
Horse 2*	10	10.5	12	8	13.5	5.5	0	8	9.5	11	0	0	7	12	18.5
Horse 3	10.5	11	12	11	10.5	2.5	11	8	7.5	7	0	0	0	0	0
Horse 4*	9	12.5	9	8	11	9	8.5	3	8	12	0	0	5	0	11
Horse 5	10	9.5	9.5	10.5	12	5	0	0	0	10	0	0	0	0	0
Horse 6*	10	11	11	7.5	10	0	0	9.5	3.5	11	0	0	0	5.5	14.5
Horse 7	9	9	10	11	7	0	0	0	0	2.5	0	0	0	0	0
Horse 8*	9.5	9	10.5	11.5	10	0	0	0	0	8	0	0	0	9.5	16.5
Horse 9*	7	9	7	8	9	0	0	0	0	4	0	0	0	0	0
Horse 10	9	8.5	8	7.5	10.5	0	0	0	0	8	0	0	0	0	0

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